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The Onset and Maturation of the Graft versus Host Reaction in Chickens

by J. B. SOLOMON[1]

From the Chester Beatty Research Institute, London

INTRODUCTION

WHEN implants of spleen from mature homologous chicken are placed on the chorio-allantoic membranes of chicken eggs, certain of the adult cells rapidly invade the vascular system of the host (Dantchakoff, 1918). Some of these cells colonize the spleen of the host embryo (Biggs & Payne, 1959; Ebert, 1959; Simonsen, 1957) and after a short latent period these adult cells proliferate in the host spleen under the stimulus of continuous exposure to the individual specific foreign antigens of the host. The resulting splenomegaly of the host embryo is generally recognized to be due to the proliferation of these immunologically competent cells. However, the possibility of enhanced mitotic division of spleen cells of the embryonic host being also partly responsible for the splenomegaly has been suggested by Biggs & Payne (1959). This type of transplantation effect has been termed the 'graft versus host' reaction (Simonsen, 1957) of which the initial symptoms are splenomegaly and hepatomegaly of the host. These are followed in some cases by the more drastic symptoms of runt disease (Billingham & Brent, 1959; Duyff, 1929; Mun, Kosin, & Sato, 1959).

In 1951 Ebert related the appearance of certain antigens during the development of the chick embryo to a small but statistically significant increase of host spleen-weight when spleens from embryos of at least 14 days of incubation were used as grafts. The spleens were implanted on to the chorio-allantoic membrane of homologous chick embryos at 8 days of incubation and the host spleen removed 10 days later. This finding is not in accord with the work of Dantchakoff (1918), Delanney (1961), and Solomon (1960*a*): these workers found that under similar conditions to those used by Ebert (1951), splenic implants from chick embryos of any age did not produce splenomegaly in the host embryo. Under these conditions of implantation Solomon (1960*a*) showed that there was a sudden onset of the ability to evoke splenomegaly of the host when the donor spleens were taken from chicks which were 5 days old post-hatching. Simonsen (1957) had previously shown that no splenomegaly occurred when blood from chicks less than 11 days old post-hatching was injected into 18-day-old

[1] *Author's address*: Chester Beatty Research Institute, Institute of Cancer Research, Fulham Road, London, S.W. 3, U.K.

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chick embryos and, more recently, Isacson (1959) obtained similar results with intravenous injection of spleen cells into 14-day-old embryo hosts.

This paper presents data on the onset and maturation of the ability of small implants of certain chicken cells to evoke splenomegaly in the embryonic host and the effect of age and sex of the donor and host on the initial stages of the graft versus host reaction as shown by splenomegaly is examined.

MATERIALS AND METHODS

White Leghorn chickens and eggs supplied by Withers & Son (Appleby Farm Ltd., Kent) were used in this investigation. Brown Leghorn fowls were obtained from the Poultry Research Centre, Edinburgh. Eggs were incubated in a Westernette incubator at 99° F. for 8 days. Spleens were taken from young chicks (2–21 days post-hatching), juvenile chickens (6–20 weeks old), and adults.

Aseptic techniques were used for the removal of the tissue from the donor and the implantation and injection methods. Implants weighing about 5 to 10 mg. (wet weight) were prepared by cutting up the tissues in a Petri dish and then gently macerating the pieces with curved forceps. The method of implantation was essentially that described by Willier (1924). A slit was cut into the shell above the air-sac and a triangular window (1 × 1 × 1 cm.) was cut in the shell (with a cutting disk on a dental drill) at a point where a large vein of the chorio-allantoic membrane had been previously located by candling. The shell membrane was punctured, the chorio-allantoic membrane dropped, and the macerated implant placed on a vein of the chorio-allantoic membrane. A sterilized cellophane square was placed over the window which was sealed with paraffin wax containing some Sudan red dye. The eggs were placed in an incubator with the window uppermost and were then incubated for a further 10 days at 99° F. Embryos were removed at 18 days of incubation, dried on paper, and weighed to the nearest 0·1 g. Livers and spleens were removed and placed on moist lint and the host embryos sexed. The gall-bladders were removed from the livers, and the latter were weighed to the nearest mg.; spleens were weighed to the nearest 0·1 mg. Each experiment included sham-operated controls which underwent the same operation as the experimental eggs except that they had no tissue implanted. Chicks used as donors were sexed by examination of their gonads. Unless stated otherwise, spleens from White Leghorn donors were implanted on to 8-day-old White Leghorn host embryos and the livers and spleens removed 10 days later, i.e. at 18 days' incubation.

Ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein were estimated by extraction and colorimetric methods similar to those used in previous work on the chick embryo (Solomon, 1957*a*). Spleens were homogenized in ice-cold phosphate buffer, pH7 (1 g./15 ml.), and a small fraction of this was retained for protein estimation. The remainder of the homogenate was brought to 0·2 M perchloric acid (PCA) with 1·0 M PCA and stirred for 15

minutes at 4° C. After centrifugation, the supernatant containing acid-soluble nucleotides was discarded and the pellet resuspended in 1·0 M PCA (approx. 5 × pellet volume) and incubated at 70° C. for 20 minutes. After centrifugation and a further similar extraction and centrifugation the pooled supernatants were retained. Protein was determined by the method of Sutherland, Cori, Haynes, & Olsen (1949), DNA was measured by the method of Burton (1956), and RNA was measured by the orcinol method of Ceriotti (1955), omitting the isoamyl alcohol extraction, but with a preliminary hydrolysis of the extract with 0·1 M NaOH at 100° C. for 15 minutes to remove interfering hexose compounds. Standards were bovine serum albumin and chick-liver RNA and DNA.

RESULTS

Splenomegaly of the host as a proliferation of cells

There is abundant histological evidence that the increase of host spleen-weight in the graft versus host reaction is due to cellular proliferation (e.g. Dantchakoff, 1918; Ebert, 1951; Biggs & Payne, 1961). There is also evidence that the increase in wet weight is accompanied by an increase in total nitrogen (Ebert, 1954). As Anreini, Drasher, & Mitchison (1955) found that a slight increase in the RNA to protein ratio apparently accompanied antibody production in mice, both RNA and DNA as well as protein and dry weight have been measured in this work. The RNA, DNA, and protein content was determined in host spleens

TABLE 1

RNA, DNA, and protein content of host spleens from chick embryos grafted with splenic implants from embryo, chick and adult donors

Donor	*Donor ages*	*Number of determinations*	*Host spleen*			
			Wet wt. (mg.)	*RNA (mg./g. wet wt.)*	*DNA (mg./g. wet wt.)*	*Protein (mg./g. wet. wt.)*
None	—	14	12·6±0·3	7·1±1·4	4·1±0·5	130±10
Embryo	15–20 days	3	13·6±1·2	5·9±1·7	3·2±0·8	144±18
Chick	8–21 days (post-hatching)	7	24·3±1·8	7·6±0·8	4·4±0·4	152±12
Adult	20–78 weeks	5	29·8±3·0	5·9±2·3	4·5±1·0	123±8

from some of the experiments to be described later. These included experiments in which the hosts received grafts of spleen from 15- to 20-day-old embryos, from 8- to 21-day-old chicks, and from adult fowls of 20–78 weeks old. Each determination of nucleic acids and protein was made in duplicate on the extract from 100 to 200 mg. host spleen. Table 1 shows that the RNA, DNA, and protein per g. wet weight remained constant in both the sham-operated control host spleens and in the non-enlarged and enlarged experimental spleens. These results do not

agree with those of Ebert (1954), who found that the DNA content per g. wet weight of the host spleens bearing adult chicken spleen implants showed a statistically significant 30 per cent. decrease below that of the controls. There was no change in the RNA/protein ratio in this work. Dry weights were determined by drying the spleen overnight at 90° C.; the dry weight, expressed as a percentage of the wet weight, was 18·3 per cent. for 7 control spleens (mean wet weight 15·6 mg.) and 19·4 per cent. for 10 selected enlarged spleens (mean wet weight 61·8 mg.) from hosts which had been implanted with adult spleen. It can be concluded that the increase in wet weight of the spleens is due to cellular proliferation. When spleen cells from competent fowls were implanted, the enlarged livers frequently appeared green due to bile stasis and the gall-bladders were shrunken; enlarged spleens usually had a normal appearance except that occasionally they showed marked white nodules.

Comparison of the effectiveness of spleen, liver, bone-marrow, and thymus in eliciting splenomegaly

Several tissues of the chicken have been found to evoke the graft versus host reaction. Table 2 shows a few experiments in which two or three tissues were taken from the same donor and small implants of each tissue were then used in the 8–18-day White Leghorn host system. The active cells are distributed in the thymus, liver, and bone-marrow as well as in the spleen; all these tissues are

TABLE 2

Comparison of the effectiveness of spleen, liver, thymus, and bone-marrow from juvenile and adult donors in eliciting splenomegaly

Donor			*Host*			
Age (weeks)	*Number*	*Tissue implanted*	*Number*	*Body mean wt. (g.)±SE*	*Liver mean wt. (mg.)±SE*	*Spleen mean wt. (mg.)±SE*
—	None	None	89	21·1±0·3	483±11	13·3±0·5
7	1	Thymus	7	†21·8±1·0	654±40	*31·9±7·1
		Spleen	9	*17·2±1·6	†655±88	30·4±4·0
9	1	Spleen	20	—	612±24	26·4±1·1
		Bone-marrow	7	—	718±37	36·0±1·5
15	1	Liver	11	†19·3±1·1	†498±38	31·0±4·4
		Spleen	17	19·4±0·4	†500±19	33·8±3·3
60 and 77	2	Liver	8	*19·2±0·6	†504±58	21·4±2·6
		Spleen	17	17·5±0·8	†518±24	45·8±3·0
		Bone-marrow	10	17·0±0·5	†490±32	23·3±3·4
35‡ and 40‡	2	Liver	7	—	—	16·9±0·9
		Spleen	17	†21·5±0·9	†505±6	†22·2±5·1
		Bone-marrow	13	†20·9±0·9	†594±55	*25·2±5·0

Difference between experimental and control weights: † no difference; * $P = 0{\cdot}05$; for all others, $P = 0{\cdot}01$.

‡ Brown Leghorn donor.

rich in reticulo-endothelial cells in the chicken. In the White Leghorn donor to White Leghorn host experiments in Table 2 liver tissue from 3 donors produced a host spleen mean weight of 27·3±2·9 mg. (19 hosts), bone-marrow from 2 donors produced a host spleen-weight of 28·5±3·3 mg. (17 hosts), thymus from one donor produced a mean spleen-weight of 31·9±7·1 mg. (7 hosts), and spleen from 5 donors produced a host spleen-weight of 34·4±3·2 mg. (63 hosts). There was no significant difference between any of these mean weights.

Onset of competence in White Leghorn spleens implanted into White Leghorn embryos

Solomon (1960*a*) reported that chicks up to 4 days post-hatching were incapable of producing splenomegaly and that there was a tendency for greater increases of spleen-weight to occur in female hosts rather than in the male hosts. These results are shown in full in Table 3 which gives results from one hatch of White Leghorn chick donors. In this case the onset of competence to produce splenomegaly is shown at 5 and 6 days post-hatching in the female hosts. In the experiment in which 6-day-old chick spleens were used as donors there was a

TABLE 3

Onset of the competence of chick spleen to elicit splenomegaly

Donor		*Host*					
		Number			*Spleen mean wt. (mg.)±SE*		
Age (days post-hatching)	*Number*	*Male*	*Male and female*	*Female*	*Male*	*Male and female*	*Female*
—	None	—	71	—	—	13·1±0·5	—
2	11	—	24	—	—	†15·9±2·2	—
4	4	3	—	6	†14·9±4·0	—	†13·7± 0·8
5	11	11	—	12	†16·3±3·0	—	*20·0± 3·3
6	7	12	—	14	†13·9±1·5	—	*32·5± 7·2
7	7	10	—	13	36·7±5·5	—	39·0± 6·8
8	6	12	—	15	*19·9±2·6	—	†36·1±15·9

Difference between experimental and control weights: † no difference; * $P = 0{\cdot}05$; for all others, $P = 0{\cdot}01$.

statistically significant difference ($P = 0{\cdot}05$) in the spleen-weights of the male and female hosts; the same tendency was shown in the experiment with 8-day-old chick donors. However, there was no difference between the male and female hosts when 7-day-old chicks were used as donors. The time of onset of competence of donor chick spleen cells to produce splenomegaly has not been so well defined in other experiments. This has been due to a lower proportion of enlarged spleens and consequently a small increase in the mean weight of the host spleens. For example, in one experiment spleens from 10-day-old chick donors produced only one enlarged spleen of 89 mg. out of 25 within the normal range. Similarly, in earlier experiments (Solomon, 1960*a*), 2-day-old chicks had

produced one enlarged host spleen of 60 mg. out of 19 others which had been within the normal (control) range of 5–25 mg., and 5-day-old chicks had produced only one enlarged spleen of 38 mg. in 23 spleens within the normal range.

TABLE 4

Inability of embryonic spleen to elicit splenomegaly

Donor		*Host*			
		Number		*Spleen mean wt. (mg.)±SE*	
Age (days of incubation)	*Sex*	*Male*	*Female*	*Male*	*Female*
—	—	10	12	11·8±0·8	12·2±0·4
15	Male and female	5	4	14·4	14·5
18	Male	—	2	—	11·4
18	Female	4	2	12·4	11·2
20	Male	6	8	7·7	9·4
20	Female	4	6	7·8	11·0
1 day post-hatching	Female	5	3	13·9	8·9
TOTALS		24	23	13·2±0·7	10·9±0·7

One donor spleen to each embryo.

TABLE 5

Ability of male chick spleen implants to elicit splenomegaly

Donor		*Host*					
		Number		*Liver mean wt. (mg.)±SE*		*Spleen mean wt. (mg.)±SE*	
Age (days post-hatching)	*Number*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
—	None	5	8	347±51	346±43	9·9±1·4	10·7±0·6
8	12	14	12	*476	†393	*15·4	18·1
11	1	5	5	*477	†411	†17·2	*21·3
15	3	10	8	*474	506	25·7	26·1
TOTALS 8–15	16	29	25	*477±14	†433±16	*19·2±1·9	21·2±2·0

Difference between experimental and control weights: † no difference; * $P = 0{\cdot}05$; for all others, $P = 0{\cdot}01$.

It was desirable to ascertain whether any small sex differences occurred when incompetent cells were used as donor tissue. Table 4 shows some small-scale experiments with 15-, 18-, and 20-day chick embryo donors supplying small implants of spleen for the 8–18-day period of host incubation. No increase in spleen-weight and no sex differences in the mean spleen-weights were observed. The sex differences in host spleen-weight which were found when spleens from unsexed chick donors were used (Table 3) were examined more fully in separate experiments using male or female donors. Table 5 shows the results of 3 experiments with male chick donors of from 8 to 15 days post-hatching. Most of the host spleen- and liver-weights showed statistically significant increases compared

with the controls; no sex differences in host spleen-weight were found. When female chicks 8–21 days post-hatching were used as donors, the 8-day-old chick spleens did not elicit hepatomegaly or splenomegaly in the hosts (Table 6). However, 11-day-old female chick donors produced a highly significant hepatomegaly

TABLE 6

Ability of female chick spleen implants to elicit splenomegaly

Donor		*Host*					
Age (days post-hatching)	*Number*	*Number*		*Liver mean wt. (mg.)±SE*		*Spleen mean wt. (mg.)±SE*	
		Male	*Female*	*Male*	*Female*	*Male*	*Female*
—	None	8	13	357±35	368±33	9·2±1·0	10·5±0·9
8	6	10	11	†396	†413	†10·6	†16·6
11	3	4	8	502	*470	23·7	25·4
15	3	8	9	†419	566	†20·1	35·1
21	2	4	4	†452	*512	*19·2	21·9
TOTAL 8–21	14	26	32	*418±18	*484±21	16·9±1·5	24·6±3·1

Difference between experimental and control weights: † = no difference; * $P = 0{\cdot}05$; for all others, $P = 0{\cdot}01$.

TABLE 7

Maturation of the competence of juvenile chicken spleen to elicit splenomegaly

Donor			*Host*			
Sex	*Age (weeks)*	*Number*	*Number*	*Mean body wt. (g.)±SE*	*Liver mean wt. (mg.)±SE*	*Spleen mean wt. (mg.)±SE*
—	—	None	109	22·0±0·5	534±9	14·5±0·4
Male and female‡	1–7	57	187	19·4±0·4	†561±38	23·5±1·0
—	—	None	137	20·5±0·7	484±7	12·8±0·3
Male	9–16	4	58	†19·8±1·2	570±24	29·0±2·1
	26–32	2	23	†20·9±0·5	661±37	34·8±4·5
	46–78	4	44	†19·5±0·6	617±18	42 ±5·2
Female	12–17	3	22	16·3±0·4	†484±21	23·1±3·2
	21–22	2	10	*17·7±1·0	*549±21	38·8±4·8
	52	2	29	17·5±0·4	*625±31	39·6±5·3

Difference between experimental and control weights: † no difference; * $P = 0{\cdot}05$; for all others, $P = 0{\cdot}01$. ‡ Data from Solomon (1960*a*).

and splenomegaly in the host. Although there were no significant sex differences in the host liver- and spleen-weights within the individual experiments, the total liver- and spleen-weights from all 4 experiments showed a statistically significant ($P = 0{\cdot}05$) increased liver- and spleen-weight in the female hosts.

Maturation of the competence to evoke splenomegaly

Earlier work by Solomon (1960*a*) had shown that spleens taken from chicks of from 1 to 7 weeks of age (post-hatching) produced a 62 per cent. increase in host spleen-weight, but no hepatomegaly (Table 7). As the age of the donor chicken increased so did the extent of the hepatomegaly and splenomegaly of the embryonic hosts (Table 7). Spleens from male donors of from 9 to 16 weeks of age gave a significantly greater ($P = 0{\cdot}05$) increase of spleen-weight above those of the 1–7-week age group. Although the 26–32-week-old male donor group did not produce significantly greater splenomegaly and hepatomegaly compared with that of the 9–16-week-old group, the 46–78-week-old donor group produced significantly greater ($P = 0{\cdot}05$) splenomegaly than the 9–16-week-old group. No significant decreases in whole body-weight of the host embryos was observed in the experiments with male donors. The female donor spleen from 12–17-week-old chickens did not produce any hepatomegaly and no greater splenomegaly of the hosts than the 1–7-week-old male and female donor group. The 21–22- and the 52-week-old female donors both produced significantly greater ($P = 0{\cdot}05$) hepatomegaly and splenomegaly than the 12–17 female donor group. Highly significant reductions of from 13 to 20 per cent. in the host body-weight were produced by female donors of from 12 to 52 weeks of age.

TABLE 8

The splenic weight of chick embryos implanted with juvenile chicken spleen

Donor			*Host*					
			Number		*Liver mean wt. (mg.)±SE*		*Spleen mean wt. (mg.)±SE*	
Sex	*Age (weeks)*	*Number*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
—	—	None	16	18	427±14	442±23	12·0±0·7	12·4±0·9
Male	6–17	12	15	22	*542±39	*532±40	27·4±3·3	28·7±2·9
Female	6	2	8	11	544	601	26·2	34·0
	13	3	21	11	502	516	22·5	24·2
	16	1	6	11	514	510	26·0	27·4
	17	1	8	4	443	541	25·5	32·3
	20	1	20	22	523	570	35·8	49·6
TOTAL FEMALE	6–20	8	63	59	508±5	560±5	27·9±2·1	36·6±3·5

Difference between experimental and control weights: * $P = 0{\cdot}5$; for all others $P = 0{\cdot}01$.

The ability of spleens from juvenile donors to produce splenomegaly in the host was re-examined with the sex of the donor and host determined. Results in Table 8 show that with male donors 6–17 weeks of age there was no sex difference in the increased spleen-weight of the hosts. However, when pieces of spleen from 6–20-week-old female donors were used as implants, the splenomegaly of the

female hosts tended to be greater than that of the male hosts by as much as 14 mg. (Table 8). Although this tendency was not statistically significant within any individual experiment with female donor spleen, the total results from experiments in which female donors from 6 to 20 weeks were used showed a significantly increased mean spleen-weight ($P = 0{\cdot}05$) and liver-weight ($P = 0{\cdot}01$) of the female hosts above that of the male hosts. Solomon (1961) has since found that large implants (30 mg.) of macerated spleen from juvenile female donors produced an increase of spleen-weight of the female hosts which showed a highly significant ($P = 0{\cdot}01$) difference from that of the other three sex combinations.

TABLE 9

The splenic weights of chick embryos implanted with adult chicken spleen

Donor		*Host*					
		Number		*Liver mean wt. (mg.)±SE*		*Spleen mean wt. (mg.)±SE*	
Sex	*Number*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
	None	24	15	423±24	376±36	12·1±0·5	11·2±1·0
Male	3	22	18	616±41	533±34	28·8±4·5	30·3±5·2
Female	4	32	25	558±37	537±25	43·5±6·1	40·8±5·8

All experimental weights are greater than control weights ($P = 0{\cdot}01$).

The absence of sex differences in the reaction in male and female host spleens when small implants of adult cockerel or hen spleens were used as donor is shown in Table 9. Body-weights of experimental embryos were only 10 per cent. below those of the controls.

DISCUSSION

The extent of the increase of spleen- and liver-weight of a chick embryo acting as host to immunologically competent homologous cells is subject to a large number of biological variables. These include the number of competent donor cells injected, the genetic diversity between donor and host (Cock & Simonsen, 1958; Isacson, 1959; Mun, Kosin, & Sato, 1959), the extent of colonization of the host spleen by donor cells (Simonsen, 1957; Cock & Simonsen, 1958), and the age of the donor cells with regard to their immunological maturity. When cells are administered as implants there may be another variable due to the extent of the invasion of implant cells into the vascular system of the host.

Dantchakoff (1918) mentioned that she had found that homologous liver and bone-marrow as well as spleen produced splenomegaly of the host embryo. Willier (1924) found that thyroid, liver, thymus, and spleen produced splenomegaly. Ebert (1954) stated that of 11 tissues studied, only spleen, thymus, and

liver were effective but that neither thymus and liver were as effective as spleen and that thymus was better than liver. However, in this work it has been shown for donors at various ages that small implants of liver, thymus, and bone-marrow are as effective at producing splenomegaly and hepatomegaly as splenic implants. Billingham & Silvers (1959) have found that adult chicken skin will also elicit splenomegaly. These results probably only mean that there are sufficient competent cells in these tissues capable of invading the hosts' vascular systems and colonizing the hosts' spleen. The concept of organ specificity due to protein templates (Weiss, 1947) does not appear to be tenable in this type of reaction as it is more probably due to a specific cell type such as the large lymphocyte (Terasaki, 1959) which can be found in several different tissues in the chicken.

Hepatomegaly, which does not always accompany splenomegaly in the initial stages of the graft versus host reaction, is presumably also produced by colonization of the liver by certain lymphoid cells from the implant. In this work hepatomegaly has usually accompanied splenomegaly; when homologous spleen cells were injected intravenously into 15-day-old chick embryo hosts only the extent of the splenomegaly increased with cell dosage (Solomon, unpublished results). Hepatomegaly and splenomegaly are sometimes accompanied by reduction in whole body-weight which may be a 'runting' effect. Such a reduction in whole body-weight has not been related to the extent of splenomegaly. Differentiation is not retarded in such runted embryos.

Biggs & Payne (1959) found by cytogenetic analysis of the enlarged chimeric chick spleens that about half of the cells in mitosis are of donor origin and that the other half belong to the host. While furnishing clear proof of colonization of intact dividing donor cells in the host spleen (cf. Ebert, 1959), the fact that mitotic division of the host spleen cells is possibly enhanced is of great interest. This reaction of the embryonic cells cannot be immunological as the embryo host is not yet competent. Biggs & Payne (1961) found that the splenomegaly syndrome lasted for 7 days after intravenous injection of adult cock spleen cells into 15-day-old chick embryos. It consisted of extensive proliferation of reticulum cell foci and a blast cell and granulocytopoietic response. Biggs & Payne suggested that the reticulum cell foci and some of the blast cells are of donor origin and that most of the blast cells and developing granulocytes may be of host origin.

As no splenomegaly occurs with non-competent (embryonic) homologous donor cells or with fully competent donor cells injected into an isologous host (Cock & Simonsen, 1958), the mitosis of the embryonic spleen is probably a secondary effect induced by the primary proliferation of colonies of neighbouring adult cells.

The cellular proliferation of competent donor cells on exposure to the tissue antigens of the host may be similar to the splenomegaly found in fowls treated with soluble antigens. Norton, Wolfe, & Crow (1950) obtained up to 75 per cent.

increases in spleen-weight of fowls which had been injected with bovine serum albumin and some correlation with spleen size and precipitin titre was found. They found that splenomegaly was more rapid in birds older than 4 weeks post-hatching. The onset and maturation of the ability to evoke splenomegaly in the graft versus host reaction shows, in chickens, some resemblance to the immunological maturation to other antigens (Buxton, 1954; Norton, Wolfe, & Crow, 1950; Wolfe & Dilkes, 1948).

The time of onset of the ability of donor cells to evoke splenomegaly in the host can be from 5 to 11 days post-hatching when the 8–18-day host system is used. This is in good agreement with the work of Simonsen (1957), who found the time of onset to be 11 days post-hatching when older embryos were injected with adult blood. In the 8–18-day host system, which is essentially the same as that used by Ebert (1951), it has been impossible to show such a well-defined time of onset except in one series of experiments on the same batch of chicks. More recent work (Solomon & Tucker, unpublished results) has indicated that this failure to obtain a well-defined time of onset in the 8–18-day host system may be due to the low extent of colonization of donor cells in the host spleen when embryos are implanted at 8 days of incubation. The onset of the ability of donor cells to evoke splenomegaly at 11 days post-hatching is in good agreement with the work on transplantation of skin grafts in the chick. Tolerance is most readily induced in embryos or newly hatched chicks, and the 'null period' between this phase and the age after which the chick will reject a skin graft occurs within the 2nd to the 7th day post-hatching (Cannon & Longmire, 1952; Billingham, Brent, & Medawar, 1956).

The student of development has difficulty in finding substances or functions which suddenly arise *de novo* during embryogenesis or later development. Some enzymes have been found to be at higher concentrations in embryonic tissues than in those of the adult (Solomon, 1957*b*, 1958) and attempts to induce synthesis of enzymes in embryos *in vivo* have so far been unsuccessful (e.g. Solomon, 1960*b*). The onset of the ability to evoke splenomegaly, while probably entirely cellular in nature, has the great virtue that it is entirely absent in embryonic life and is a clear example of a sudden acquisition of function during post-embryonal development.

Data in this work show that this ability increases with the age of the chicken, reaching a maximum at about 20 weeks post-hatching. These results are somewhat different to those of Ebert (1951), who claimed that the 6-week-old chicken spleen was mature in this respect although it and adult chicken spleen only produced a twofold increase in host spleen-weight. However, Ebert later (1954) obtained greater (fourfold) increases in host spleen-weight with adult cockerel donors, and this is in agreement with the present results. A similar maturation effect has been found in the homograft reaction in mice (Mariani, Martinez, Smith, & Good, 1959).

The small differences in the extent of splenomegaly between male and female

hosts which have been found in this work have, as yet, no ready explanation. They are only apparent when young chick or juvenile female spleens are used as donor tissue and the reaction in the female host is greater than in the male hosts. The female-female donor-host combination in experiments using 8–21-day-old chick donors (Tables 5, 6) gave a mean spleen-weight of 24·6 mg., which was not significantly greater than that of the other three sex combinations when they were pooled (range of mean spleen-weights 16·9–21·2 mg.). However, in experiments with 6–20-week-old juvenile chicken donors (Table 8) the female-female donor-host combination gave a mean spleen-weight of 36·6 mg., and this was significantly greater ($P = 0{\cdot}02$) than those of the other three sex combinations when they were pooled (range of mean spleen-weights 27·4–28·7 mg.). The finding that, under certain conditions, female hosts bear greater spleens than male hosts, may have a hormonal basis, and the enhancement of the splenomegaly syndrome by female hormone is being investigated.

SUMMARY

1. The increase in wet weight of the host spleen following implantation of competent chick, juvenile or adult chicken spleen has been shown to be paralleled by increases in dry weight and in content of RNA, DNA, and protein.

2. No increase in the spleen-weights of the hosts was found when non-competent spleen cells from chick embryos or 1-day-old chicks were used as donor.

3. The onset of immunological competence with respect to this transplantation reaction has been shown to occur at about 5 days post-hatching when the spleens were implanted into embryos at 8 days of incubation and the host spleens removed 10 days later.

4. The ability of chicken spleen cells to evoke hepatomegaly and splenomegaly of the embryo hosts increased with age and was greatest after 21 weeks of age. Sometimes a reduction in whole body-weight of the embryo hosts also occurred.

5. Although no sex differences in host spleen-weight were observed when 8–15-day-old male chick spleens were used as grafts, a significant increase in female host liver- and spleen-weights (compared with those of the male hosts) was noted when 8–21-day-old female chick spleens were used as grafts.

6. Spleen cells from male chickens of 6–17-weeks old did not produce much greater splenomegaly than those from young chicks and there were no sex differences between the host embryo spleen-weights. However, when female spleen tissue from 6- to 20-week-old chicks was used as graft a significant increase in the hepatomegaly and splenomegaly of the female hosts above those in the male hosts was observed.

7. No sex differences were obtained in embryo host spleen-weights when small implants of adult male or female spleen cells were used as grafts.

8. Thymus, liver, and bone-marrow from competent donors have been found

to be nearly as effective as spleen in producing splenomegaly of the embryo hosts.

RÉSUMÉ

Apparition et réalisation de la réaction anti-hôte du greffon chez le Poulet

1. L'augmentation du poids brut de la rate de l'hôte après implantation de rate compétente de poulet jeune ou adulte correspond à une augmentation de son poids sec et de sa teneur en ARN, ADN et protéines.

2. On n'a pas observé d'augmentation du poids de la rate de l'hôte quand on utilise comme greffon des cellules spléniques non compétentes d'embryon ou de poussin à l'éclosion.

3. On a montré que l'apparition de la compétence immunologique en ce qui concerne cette réaction de transplantation se situe au 5e jour environ après l'éclosion, si on a implanté les rates dans des embryons au 8e jour de l'incubation, puis ôté la rate de l'hôte 10 jours plus tard.

4. L'aptitude des cellules spléniques de poulet à provoquer l'hépatomégalie et la splénomégalie de l'embryon-hôte a augmenté avec l'âge, et a été maximale après l'âge de 21 semaines. Quelquefois aussi est survenue une diminution du poids corporel total des embryons-hôtes.

5. Bien qu'on n'ait pas observé de différences selon le sexe dans le poids de la rate des hôtes quand on a utilisé comme greffons des rates de poussins mâles âgés de 8 à 15 jours, on a noté un accroissement significatif du poids du foie et de la rate des hôtes femelles par rapport à celui des mâles quand on a utilisé comme greffons des rates de poussins femelles âgés de 8 à 21 jours.

6. Des cellules spléniques de poulets mâles de 6 à 17 semaines n'ont pas provoqué une splénomégalie beaucoup plus importante que ne l'ont fait celles de jeunes poussins, et il n'y avait pas de différences d'ordre sexuel entre les poids des rates des embryons-hôtes. Néanmoins, quand on a utilisé comme greffon une rate de femelle âgée de 6 à 20 semaines, on a observé une augmentation significative de l'hépatomégalie et de la splénomégalie des hôtes femelles, par rapport à celles des hôtes mâles.

7. On n'a pas décelé de différences selon le sexe dans le poids des rates des embryons-hôtes quand on a utilisé comme greffon de petits implants de cellules spléniques d'adultes mâles ou femelles.

8. Le thymus, le foie et la moelle osseuse de donneurs compétents sont presque aussi actifs que la rate pour produire la splénomégalie des embryons-hôtes.

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The Importance of Mesenchymal Factors in the Differentiation of Chick Epidermis

I. The Differentiation in Culture of the Isolated Epidermis of the Embryonic Chick and its Response to Excess Vitamin A

by C. B. McLOUGHLIN[1]

From the Strangeways Research Laboratory, Cambridge

WITH FOUR PLATES

INTRODUCTION

It is well established that in the developing chick the underlying mesenchyme initiates the appearance of specific epidermal derivatives, e.g. feathers (Sengel, 1956), claws (Cairns & Saunders, 1954), and the preen gland (Gomot, 1958). On the other hand, it is not yet known to what extent the epidermis is independent of mesenchymal intervention for its basic differentiation into a stratified, squamous epithelium. Sobel's (1958) work on the 8-day chick pituitary suggests that the differentiation and multiplication of certain epithelial cells cannot proceed in the absence of mesenchymal elements. She found that the isolated epithelial cells of the hypophysial rudiment survived but were unable to differentiate or multiply; when associated with perichondrial fibroblasts, however, they resumed mitosis and produced typical α and β cells.

In the first part of the present investigation, experiments were made to see whether the embryonic epidermis, like the hypophysial epithelium, requires the presence of fibroblasts to enable it to grow and differentiate, or whether it can proliferate, acquire its characteristic squamous structure and keratinize, when isolated and cultivated in the absence of connective tissue.

It has been shown (Fell & Mellanby, 1952; Fell, 1957) that when embryonic chicken skin is cultivated in medium containing excess vitamin A (7·5–22·5 i.u./ml. of medium), the epidermis fails to keratinize and is transformed into a columnar, mucus-secreting epithelium which sometimes becomes ciliated. In view of the fact that excess vitamin A also produces striking changes in the intercellular material of cartilage (Fell & Mellanby, 1952; Fell & Thomas, 1960), it seemed possible that in the skin explants the action of the vitamin might be primarily on the dermal connective tissue and that the epithelial metaplasia might be a secondary effect.

[1] *Author's address:* Strangeways Research Laboratory, Wort's Causeway, Cambridge, U.K.

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The second part of this study was undertaken to test this hypothesis; the effect of excess vitamin A on isolated epidermis in culture has been examined, to find whether the mucous change would appear in the absence of the dermal fibroblasts.

The results show that the isolated epidermis is capable of keratinizing and of responding to excess vitamin A by mucus secretion, but that it cannot grow in the absence of mesenchyme.

MATERIAL AND METHODS

Material

For a histological study of the normal differentiation of chick skin *in vivo*, the entire limb-buds of 4- and 5-day embryos and fragments from the thigh and shank regions of 8-, 10-, 12-, 13-, and 17-day embryos were used.

Cultures were made from the skin of 5-day limb-buds.

Tissue culture

Cultures of intact skin. The development in culture of the intact skin was studied in 26 explants fixed at intervals of 1 to 11 days. The tissue was grown by Maximow's double coverslip method in a medium composed of 2 parts of cock's plasma and 1 part of embryo extract; the extract consisted of 1 part of embryo mince (13-day chick) and 2 parts of Tyrode's solution. The explants were fed with fresh medium after 2 days' growth and every 4th day were transplanted to a fresh coverslip, a procedure that prevented undue spreading of the tissue.

Cultures of isolated epidermis. Epidermis was isolated from the mesenchyme as follows. The hind limb-buds were removed from the 5-day embryo and their apical ridges cut off and discarded; the rest of each bud was cut transversely, but somewhat obliquely, into 4 wedge-shaped pieces which were washed for 4 minutes in Ca–Mg-free Tyrode's solution and then transferred to a 3 per cent. solution of crude trypsin (BDH) in Ca–Mg-free Tyrode (Moscona, 1952). After about 10 minutes the epidermis began to separate from the mesenchyme; the process was hastened by gently rocking the dish containing the fragments, and completed by teasing the delicate sheets of epidermis free with fine, blunted glass needles. As each sheet of epidermis was detached, it curled into a tight roll with the periderm outwards; to inactivate the trypsin these rolls were transferred to a dish of horse serum diluted with an equal volume of Tyrode. After 15 minutes in the serum, the fragments were explanted on Maximow double coverslips in a mixture of 2 parts plasma and 1 part embryo extract; the epithelium degenerated when a higher concentration of extract was used. The explants were fed and transplanted as described above, and were fixed after 1 to 10 day's cultivation. Sixty-two explants of isolated epidermis grown in normal medium were examined.

During tryptic digestion a sticky transparent mucoid material oozed out of the mesenchyme and adhered firmly both to the mesenchyme cells and to the

epithelium (Plate 1, fig. 1), with the consequence that the isolated epidermis sometimes carried with it a wisp of this material through the entire procedure to explantation. On prolonged exposure to trypsin the mucoid gradually dissolved. This is the same intercellular material whose importance to the reaggregation of separated cells has been established by Moscona (1961). As described below, it was also found to affect the orientation of epidermal cells.

For the experiments with vitamin A, synthetic vitamin A alcohol was dissolved in ethanol and added to the plasma so as to give a concentration of either 2·5 or 6·6 i.u. vitamin A/ml. in the culture medium. The solution of vitamin A in ethanol was always such that the concentration of ethanol in the plasma was 0·02 per cent. An equal amount of ethanol was added to the plasma of the control cultures. Eighteen explants were grown in vitamin A medium.

Histology

Tissues were fixed in 3 per cent. acetic Zenker's solution or in Carnoy's fluid, and sections were stained with Meyer's acid haemalum and eosin or alcian blue (0·5 per cent. in distilled water, 20 seconds), by Mallory's azan method, Heidenhain's iron haematoxylin, or the periodic-acid/Schiff method (PAS).

RESULTS

Normal development in vivo *of the limb-bud epidermis*

The epidermis of the limb-bud of a 5-day chick embryo is a delicate 2-layered structure composed of an outer periderm (epitrichium) and an inner basal layer (Plate 1, fig. 2).

The basal cells, which will finally produce the entire keratinizing epidermis, are seated on an extremely thin basement membrane which stains with the PAS technique. They are stellate and arranged in a single irregular layer between the basement membrane and the periderm (Plate 1, figs. 2, 3); their mitotic rate is high.

The cells of the periderm are extremely flattened and can be seen best in oblique sections (Plate 1, fig. 3); they are capable of division and so, unlike the more distal layers of older epidermis, they may be independent of the basal layer for their replenishment. A periderm is present in chick embryos from at least as early as 2 days' incubation until it is sloughed from the stratum corneum in the 18-day embryo. By contrast with the stellate structure of the basal layer, the peridermal cells are firmly attached to each other, so that from the time of their appearance to about 12 days, when the gaps between the basal cells are finally closed, the periderm is the only uninterrupted cytoplasmic barrier interposed between the interior of the embryo and the amniotic fluid.

In several places the epidermis is thickened and its cells assume a typical columnar or cuboidal epithelial arrangement. These areas of epithelial thickening

are the apical ridge whose important morphological function is well known (Saunders, 1948), an axillary columnar thickening which participates in the moulding of the axilla (Saunders, 1953), and a cuboidal tract which runs along the anterior and posterior borders of the limb-bud and is continuous with both the axillary and apical thickening. This last becomes obvious by its greater rigidity when the epidermis is removed from the mesenchyme. These characteristics are better observed in 4-day limb-buds than in those of 5 days in which the epidermis has become much thinned owing to the rapid growth of the rudiment. The presence of these epithelial thickenings is correlated with localized thickenings of the basement membrane, which are both observed under the microscope and inferred from the fact that these regions are the last to be freed by tryptic digestion during the separation of the epithelium from the mesenchyme.

Till 12 days *in vivo* the epidermis remains 2-layered, since all its expansion is at first absorbed in covering the rapidly growing limb, and is later localized in the feather-germs which appear at about the 7th day. The basal layer gradually assumes a typical columnar epithelial arrangement, but the gaps between the originally stellate cells are not finally closed till the 12th day. From this stage onwards the epidermis begins to form squamous layers, but even at 17 days true keratinized epithelium, in which the nuclei have completely disappeared, is not present in thigh skin though there is a very thin layer on the scales of the shank. A stratum granulosum is generally thought to be absent from chick epidermis, but a thin layer of flattened cells containing rounded granules which take up Meyer's acid haematoxylin is visible in oblique sections of 17-day thigh epidermis.

The periderm remains microscopically unchanged until at least the 13th day. At 17 days it overlies thick epidermis on the point of becoming keratinized, and its cells contain confluent rodlets of a material that stains strongly with azocarmine and is birefringent in polarized light. As Kingsbury, Allen & Rotheram (1953) point out, this material seems to be identical with the trichohyalin of the inner root sheath of hairs. It is the characteristic product of the final differentiation of periderm.

In the thigh skin of the 12-day-old embryo, the epidermal component of the feather-germs grows and thickens vigorously; it is associated with a thin basement membrane and with the cellular mesenchyme of the dermal papilla which contains only few collagen fibrils. This contrasts sharply with the skin between the germs: here the epidermis proper grows relatively so slowly that it remains only two layers thick, and is associated with a conspicuous basement membrane and a thick dermis. This difference may reflect the epithelio-mesenchymal interaction in feather formation described by Sengel (1956), and recalls the observation of Kingsbury, Allen, & Rotheram (1953), that, in the developing chick beak, rapid growth in the epidermis is often associated with a thin basement membrane.

Differentiation of whole 5-day chick skin in vitro

The differentiation *in vitro* of embryonic chick skin grown in normal medium has been described by Miszurski (1937), Litvac (1939) and more completely by Fell & Mellanby (1953); these earlier observations were confirmed in the present experiments. The explants of 5-day chick skin usually began to produce keratin between the 5th and 8th days *in vitro* (Table 1).

TABLE 1

Differentiation in vitro *of 5-day chick skin*

Age (days)	*Number of explants*	*Undifferentiated*	*Stratified squamous*	*Keratinized*
0–3	9	7	2	—
4–6	7	2	3	2
7–11	10	1	3	6

In chick skin the details of keratinization are similar to those in mammalian skin (Plate 1, fig. 4). Tonofibrils and intercellular bridges are conspicuous, and spiral filaments of Herxheimer are present. These filaments are strong, spiral tonofibrils which usually attach the cell containing them to the basement membrane by an 'end foot'. They and their end-feet are very distinct in whole-mounts of thin sheets of skin (Plate 2, figs. 5 *a* & *b*). A stratum granulosum is sometimes seen in tangential sections as a single very flattened layer of cells beneath the keratin.

Keratin is formed faster in the explants than in the normal animal: in the embryo the 5-day epidermis takes a further 12 days to keratinize, but *in vitro* the same epidermis produces keratin in 5 to 8 days. The skin of the 5-day chick embryo seems to be under some tension, because immediately after explantation the originally stellate basal cells of the epidermis draw together to assume a typically epithelial arrangement, and the epidermal sheet retracts. In consequence the basal layer becomes columnar and the periderm cuboidal, while the basement membrane becomes very distinct (Plate 2, fig. 6). Thus, within 12 hours the basal layer of the 5-day-old epidermis has assumed an arrangement normally found only in the 12-day embryo. This probably explains its precocious keratinization *in vitro* since once the basal layer has become columnar, newly formed cells move upwards from it to give cuboidal and then squamous layers, and the epidermis begins to keratinize. Both *in vivo* and *in vitro* this takes a further 5 days, so that keratin is produced at 17 days by the living animal, but after only 5 days *in vitro* by explants.

Cells that have moved out of the basal layer into more distal strata show no sign of keratinization while they remain attached to the basement membrane by a cytoplasmic process, but as soon as they lose this contact they become squamous and begin to keratinize. The implication that keratinization can

begin only when cells lose contact with the basement membrane assumes some interest in view of the results reported later in this paper.

As Miszurski observed, the epidermis tends to form cysts in which the basal layer of the epithelium is outermost, and which keratinize inwards and are surrounded by fibroblasts. Evidence presented in the second paper of this series suggests that the dermal fibroblasts are responsible for this behaviour.

The periderm becomes cuboidal in culture so that its differentiation is more easily observed than in sections of skin from the normal animal. The cells are joined at their outer surface by terminal bars, and remain unchanged in appearance until the underlying epidermis begins to form keratin. Then the nucleus becomes crenated and the stainable cytoplasm shrinks back on to the cell membrane; the apparent perinuclear space so created fills with increasing numbers of confluent droplets of trichohyalin (Plate 2, fig. 7). Occasionally, when the epidermis keratinizes very rapidly, the periderm seems not to have time to form trichohyalin, but instead secretes a small amount of a peculiar material which differs from normal trichohyalin in the following properties: it is eosinophilic, PAS-positive, stains for abundant disulphide and sulphydryl groups with Mercury orange (Bennett & Watts, 1958), is not birefringent in polarized light, and is so hard that it scratches the microtome knife during the preparation of sections. It resembles normal trichohyalin only in staining brilliantly red with azan.

Keratinization of isolated epidermis

When the epidermis is isolated with trypsin the basal cells immediately assume a typical cuboidal epithelial arrangement, which entails some contraction of the basal layer relative to the periderm. This accounts for the curling of the epidermis, basal side in, as soon as it is separated, and supports the view that the epidermis of the 5-day chick embryo is normally under tension.

When explanted on to a Maximow double coverslip the isolated epidermis usually does not spread on the glass, but loses its 2-layered arrangement (Plate 2, fig. 8) and rounds up into a nodule which becomes completely disorganized (Plate 2, fig. 9). Many degenerations occur, probably as a result of the technical procedure. The dead material is phagocytosed during the first 24 hours by the peridermal cells, which become greatly distended and 'mark' themselves by the load of cell debris they carry, so that basal and peridermal cells can be distinguished subsequently for several days *in vitro*. During the first 12 hours the surviving cells lose contact with each other and lie scattered at random among the degenerating cells. Then they re-establish contact through fine cytoplasmic processes which extend in all directions, and draw together, so that by 48 hours they have re-aggregated into a compact epithelial ball in which an outer coat of peridermal cells surrounds concentrically arranged cells of basal origin. This segregation of the two types of cells probably occurs because only the basal cells perform the active movements of aggregation; they draw together

into a central nodule, so that the peridermal cells are passively left as an outer layer.

The peripheral basal cells have become squamous by the 3rd day, and begin to form keratin by about the 5th day *in vitro* (Plate 3, fig. 10). By the 9th day even the innermost cells have keratinized so that no living cells remain (Plate 3, fig. 11). The peridermal cells do not keratinize, but become filled with trichohyalin droplets and die. As Table 2 shows, this sequence was observed in all 62 cultures of isolated epidermis.

TABLE 2

Differentiation of isolated epidermis

Age (days)	*Number of cultures*	*Living cells present*	*Stratified squamous*	*Keratin formed*
1–3	13	13	10	—
4–7	36	31	11	25
8–10	13	—	—	13

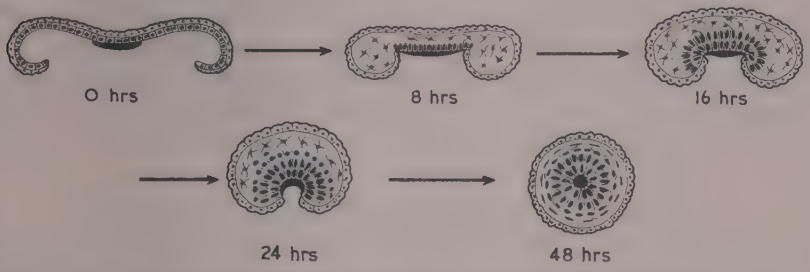
Mitoses are remarkably rare; only 4 were found in all 62 cultures. In 3 additional cultures the explanted epidermis spread out in a thin layer on the glass coverslip. None of these was kept long enough to see if the epidermis would keratinize, but only 1 mitosis was seen in all 3 cultures. Six explants of isolated gastric epithelium from 5-day chick embryos, which were made as controls, all spread on the glass and continued to secrete mucus, but once again mitoses were absent, and after 8 to 9 days in culture the epithelium usually degenerated.

Many of these epidermal cultures have in their centre a wisp of extracellular material which stains blue with azan and red with the PAS technique, and which is free from collagen fibrils detectable in the light microscope. The whole epithelial nodule is concentrically arranged around this core of mucopolysaccharide-containing material; the cells in contact with it are sharply oriented towards it in a cuboidal epithelial arrangement, and are the last to remain unkeratinized (Plate 3, fig. 12). Fig. 11 of Plate 3 also shows a small accumulation of this material at the heart of a completely keratinized nodule. Of 48 explants suitably stained for the demonstration of mucopolysaccharides, 24 were arranged around a strand of mucoid.

This is the same mucoprotein material that emerges from the mesenchyme in the course of tryptic digestion as already described. Especially after brief enzymic treatment a wisp of the mucoid often remains attached to the isolated epidermis, and its fate in becoming incorporated into the epithelial nodule suggests that it attracts the cells.

To investigate this possibility, 32 additional fragments of epidermis were removed rapidly from mesenchyme so that each carried a wisp of mucoid. They were explanted, and fixed at 8-hour intervals during the first 48 hours.

Histological study of these cultures indicated that the mucoid affects the orientation of the epidermal cells. While the rest of the explanted epidermis rounds up and becomes disoriented during the first 12 hours, the basal cells in contact with the mucoid retain their regular epithelial arrangement and become columnar (Plate 3, fig. 13). Their nuclei are aligned and their cytoplasm contains fine tonofibrils arranged at right angles to the mucoid; i.e. the cells are oriented towards this intercellular material just as they would be towards a normal basement membrane (Plate 3, fig. 14). The material obviously attracts the cells since finally they surround it completely, though at the time of explantation one



TEXT-FIG. 1. Diagram illustrating the reaction during the first 48 hours of isolated epidermis to cell-free intercellular material with which it is explanted. 0 hours: the 2-layered epidermis carries a wisp of intercellular material (black) in contact with part of the basal layer, and curls with the basal side in and the peridermal layer out. 8 hours: the basal cells have become stellate and moved apart, but those in contact with the mucoprotein retain their oriented epithelial arrangement. The outer peridermal layer remains intact. 16 hours: orientation spreads from the cells in contact with the mucoprotein to the more distant stellate cells. 24 hours: all but the most distant cells are now oriented around the mucoprotein. 48 hours: the entire epidermal nodule is oriented around the central wisp of mucoprotein, and surrounded by a coat of periderm cells.

side only of the strand is in contact with the epidermis. Gradually the orientation radiates to neighbouring cells, probably through the numerous fine cytoplasmic processes by which these cells establish contact with each other (Plate 3, fig. 15). The more distant cells are then attracted towards those originally oriented and become arranged as a second epithelial layer around them (Plate 4, fig. 16). At the same time they transmit their own orientation to other, yet more distant, cells, which in turn are attracted until all the living cells are arranged in a tight epithelial ball around the mucoid (Plate 3, fig. 12). This sequence of events is represented diagrammatically in Text-fig. 1.

These young explants of epidermis in contact with mucoid show an average of 4 mitoses per culture (Plate 3, fig. 14). This, however, cannot be attributed with certainty to a mitogenic effect of the intercellular material, as these cultures are considerably younger than those described above. Their mitotic activity may well be the delayed result of an influence exercised by the mesenchyme before the epithelium was separated from it.

The ability to orient the epidermis is peculiar to mucoprotein intercellular material and is not shared by the glycoproteins of the plasma clot. In 10

fragments of epidermis that were explanted wrapped around rayon or cotton threads, the cells were quite unaffected by contact with the foreign material.

Clearly the mucoprotein derived from the partial digestion with trypsin of the intercellular material and basement membrane is capable of directing the orientation of epidermal cells, but cultures of isolated epidermis in which no mucoid is included can also aggregate successfully to form an epithelial nodule. Epidermal cells therefore have an intrinsic tendency to aggregate and become oriented with respect to each other. The importance of the mucoid lies in the fact that, when present, it can direct the orientation of the epidermis.

That orientation is normally conferred on the epidermal cell by contact with its neighbours and with the basement membrane or substratum, is confirmed by the fate of the few single cells in each culture that had become separated from the main explant. These isolated cells lost their polarity, e.g. their tonofibrils ran circularly round the nucleus, and they accumulated fluid in an uncontrolled way until they became greatly distended (up to 100μ).

Response of isolated epidermis to treatment with vitamin A

Once it was established that the isolated epidermis keratinizes *in vitro*, two groups of explants were grown in media to which 2·5 and 6·6 i.u. of vitamin A per ml. had been added, and their differentiation was compared with that of a control group grown in normal medium.

The early behaviour of the vitamin-A-treated epidermis and the controls is very similar, i.e. both undergo rearrangement into nodules with central cells of basal origin and an outer coat of periderm, but by the 3rd or 4th day there is an obvious difference between the two groups. At a stage when a squamous arrangement has begun to appear in the controls none is to be seen in the vitamin-A-treated cultures (Plate 4, fig. 17), and a thin line of mucus appears around the outside of the ball of cells. This increases in quantity with time, and seems to be formed by both peridermal and basal cells (Plate 4, fig. 18). About the 5th day some of the outer peridermal cells of the vitamin-A-treated cultures are found to bear short cilia.

TABLE 3

Mucus formation by isolated epidermis treated with excess vitamin A

Concentration excess vitamin A (i.u./ml.)	*Age (days)*	*Number of cultures*	*Differentiation*		
			Keratin	*Mucus*	*Cilia*
0	3–7	15	15	—	—
2·5	5	6	1	—	—
6·6	3–7	12	—	9	4

Throughout the culture period of 7 days cells die occasionally and are squeezed out into the medium. These cells are not replaced as the mitotic rate is very low

(2 mitoses in 21 cultures). By the 6th day the death-rate is increasing, and on the 7th day it is clear that the cultures are gradually degenerating. The cause of death, however, is not the same as in the controls, in which all the cells keratinize.

These results which are summarized in Table 3, show clearly that the epidermal cells are capable of responding to vitamin A without the intervention of the mesenchyme.

DISCUSSION

The fact that the 5-day embryonic epidermis isolated in culture keratinizes completely, indicates both the extent and the limitation of its autonomy. Not only is the tissue able to keratinize, but it does so to an abnormal extent, i.e. including the basal layer. On the other hand, mitosis is very rare in the isolates. This can hardly be due to inadequacy of the medium because the epidermis of whole skin cultivated in the same medium grows profusely. It might be thought that the arrangement of the isolated epidermis in a ball enclosed by layers of keratin might reduce mitosis by obstructing the supply of nutrients to the inner, basal cells; mitosis is also absent, however, in the three explants that spread in a thin sheet on the glass, and is very scarce in the vitamin-A-treated explants which do not keratinize. Thus it seems probable that in normal embryonic skin the epidermis survives only because the dermis promotes mitosis and inhibits keratinization in the basal layer.

Observations on early epithelial rudiments of many different types indicate a general principle that embryonic epithelia depend on mesenchymal co-operation for their normal growth. Thus the following embryonic epithelia fail to grow in the absence of mesenchyme: the epidermis normally destined to form the preen gland of ducks (Gomot, 1958); the epithelium of the anterior pituitary rudiment (Sobel, 1958); thymus epithelium from mouse embryos (Auerbach, 1960); the gastric epithelium of 5-day chick embryos (see p. 376); and the epithelium of the submandibular gland (Dawe, personal communication). Differentiation as well as growth is inhibited in preen gland and anterior pituitary epithelium, and in the isolated epithelium of the adult mouse mammary gland (Lasfargues, 1957). On the other hand, contact with mesenchyme induces growth in the epithelium of feather germs (Sengel, 1956) and of the salivary gland rudiment (Borghese, 1950; Grobstein, 1953). Probably not all epithelia depend on association with mesenchyme for a stimulus to mitosis, since several apparently pure strains of non-malignant epithelial cells have been established in culture; in early embryonic development, however, this dependance seems to be a widespread phenomenon.

In view of its situation the basement membrane seems likely to be involved in the relation between epithelium and mesenchyme, and indeed, at sites of active growth, its structure may differ from that in less active regions. For example, in the developing beak of the chick, Kingsbury, Allen, & Rotheram (1953)

found that 'in areas of high biologic activity where there are many mitoses in both the basal cells and the adjacent mesenchyme, there appear to be few, if any, reticular fibres separating the epithelium from the connective tissue cells. It was not until shortly before hatching, when there was a thick stratum corneum, that a conspicuous basement membrane was present. The inference seems clear that there is an interaction of the adjacent epithelium and the underlying vascular mesenchyme.' Similarly, a basement membrane is absent at the point of outgrowth of the limb-bud in *Triton* (Balinsky, 1956), and as reported here, the mesenchyme and basement membrane differ in appearance in the skin proper and in the early feather germs of the chick. These observations all suggest that an alteration in the structure of the basement membrane may promote the mitogenic influence of the mesenchyme on the epithelium.

The details of the orientation-reaction of epidermal cells to cell-free intercellular material derived from the mesenchyme, are reported here for the light they may cast on the mesenchymal factors involved in determining the architecture of an epithelium. Epidermal cells clearly have a strong attraction for each other and are also specifically attracted by the intercellular material of the connective tissue, to which they react as to a basement membrane, by becoming oriented towards it and arranged around it in an epithelial manner. This behaviour represents one aspect of Moscona's generalization that 'extracellular material' is necessary for the reaggregation of dissociated cells (Moscona, 1961). The factors that normally control the orientation of epithelial cells must play an important part in their functioning and probably also in their differentiation. Epithelial cells usually line body-spaces and regulate transport between the tissues and these spaces. To do this they have to be oriented, and indeed their inner and outer surfaces differ (Ussing, 1960). Chambers (1940) has pointed out that a basement membrane is necessary for functional polarization in kidney tubules, and that mucopolysaccharide-containing surface coats orient cells in early invertebrate embryos. In the area pellucida of the avian blastodisc the single-layered cuboidal epithelial epiblast is formed against the richly PAS-positive yolk membrane, apparently by an attraction of the cells to the membrane (Jacobson, 1938). Ussing finds that only the basal layer of frog skin, i.e. that in contact with the basement membrane, is capable of active transport. This evidence combined with the observations reported here indicates that a basement membrane or surface coat is of great importance in determining the orientation and thus the normal functioning of epithelial cells. The intercellular material can also affect differentiation directly, for Wilde (1961) has shown that it is essential for, and able to alter, the differentiation of cells from *Ambystoma maculatum* neurulae.

The chemical relationship between the 'extracellular material' of early embryos and the mucoid from 5-day chick limb-buds that is capable of orienting epidermis is uncertain. The latter is a digestion product of the basement membrane and the intercellular material of the mesenchyme, and thus may contain

mucopolysaccharides, collagen, and other proteins. It stains for mucopolysaccharides, i.e. red with PAS and blue with Azan, but contains no collagen fibrils visible in the light microscope. In the absence of an electron-microscope study it is impossible to determine whether submicroscopic collagen fibrils are present.

Ozzello & Speer (1958) found that in the normal and cancerous human mammary gland, the amount of acid mucopolysaccharide in the connective tissue closely parallels the growth activity of the epithelium. Moreover, a purely epithelial strain of mammary carcinoma cells in tissue culture depends on the presence of acid mucopolysaccharides (hyaluronic acid and chondroitin sulphate C) to grow and spread, and even for survival (Ozzello *et al.*, 1960). Digestion products of these mucopolysaccharides of low molecular weight had very little growth-promoting effect. The observation mentioned in this paper that epidermal cells will not become oriented towards intercellular mucoprotein material that has undergone prolonged tryptic digestion also indicates the importance of large molecular size in the biological function of intercellular material.

The experiments on the influence of excess vitamin A on isolated epidermis in culture, show clearly that the inhibition of keratinization and subsequent mucous transformation described by Fell & Mellanby (1952) is not a secondary effect due to changes in the connective tissue, but a primary action of the vitamin on the epithelium.

From the results described in this paper, it may be concluded that at 5 days the embryonic epidermis possesses the complete equipment for keratinization, its most characteristic form of differentiation, and also for mucus-secretion in response to vitamin A. On the other hand, there is evidence of a close dependance of the epithelium on the mesenchyme in several important respects; thus the epidermal cells are unable to multiply in the absence of the dermis which also controls their orientation, and modifies their basic tendency to immediate keratinization.

SUMMARY

1. The normal differentiation *in vitro* of whole skin from the limb-bud of the 5-day chick embryo is described briefly.

2. Epidermis isolated by tryptic digestion from the 5-day limb-bud and cultivated alone *in vitro* shows very few mitoses and keratinizes completely within 10 days.

3. Isolated epidermis grown *in vitro* in a medium containing excess vitamin A fails to keratinize, secretes mucus and sometimes becomes ciliated; mitoses are rare.

4. Isolated epidermis explanted in contact with cell-free intercellular material from the mesenchyme becomes oriented towards it and arranged around it in an epithelial manner.

5. These findings indicate that while the embryonic epidermis can differentiate in the absence of mesenchyme, contact with a basement membrane and the proximity of mesenchyme and its intercellular material are required for the normal growth and functioning of epithelial cells.

RÉSUMÉ

L'importance des facteurs du mésenchyme dans la différenciation de l'épiderme de Poulet

I. *La différenciation, en culture, de l'épiderme isolé de l'embryon de Poulet et sa réponse à un excès de vitamine A*

1. Description brève de la différenciation normale *in vitro*, de la peau du bourgeon de membre d'embryon de Poulet de 5 jours.

2. L'épiderme du bourgeon de membre de 5 jours, isolé à la trypsine et cultivé seul *in vitro*, montre très peu de mitoses et une kératinisation complète en l'espace de 10 jours.

3. L'épiderme isolé, cultivé *in vitro*, dans un milieu contenant un excès de vitamine A, ne se kératinise pas, sécrète du mucus et devient quelquefois cilié; les mitoses sont rares.

4. L'épiderme isolé, explanté au contact du seul matériel intercellulaire du mésenchyme, oriente sa différenciation par rapport à lui et s'organise autour de lui à la manière d'un épithélium.

5. Ces résultats montrent que l'épiderme peut se différencier en l'absence de mésenchyme, alors que le contact avec une membrane basale et le voisinage immédiat de mésenchyme et de son matériel intercellulaire sont nécessaires à la croissance normale et au fonctionnement des cellules épithéliales.

ACKNOWLEDGEMENTS

Grateful acknowledgement is offered to Dr. H. B. Fell, F.R.S., Director of the Strangeways Research Laboratory, and to Dr. W. Jacobson for continued help and many stimulating discussions. The author also wishes to thank the Medical Research Council for a Research Scholarship, and for defraying the expenses of this work.

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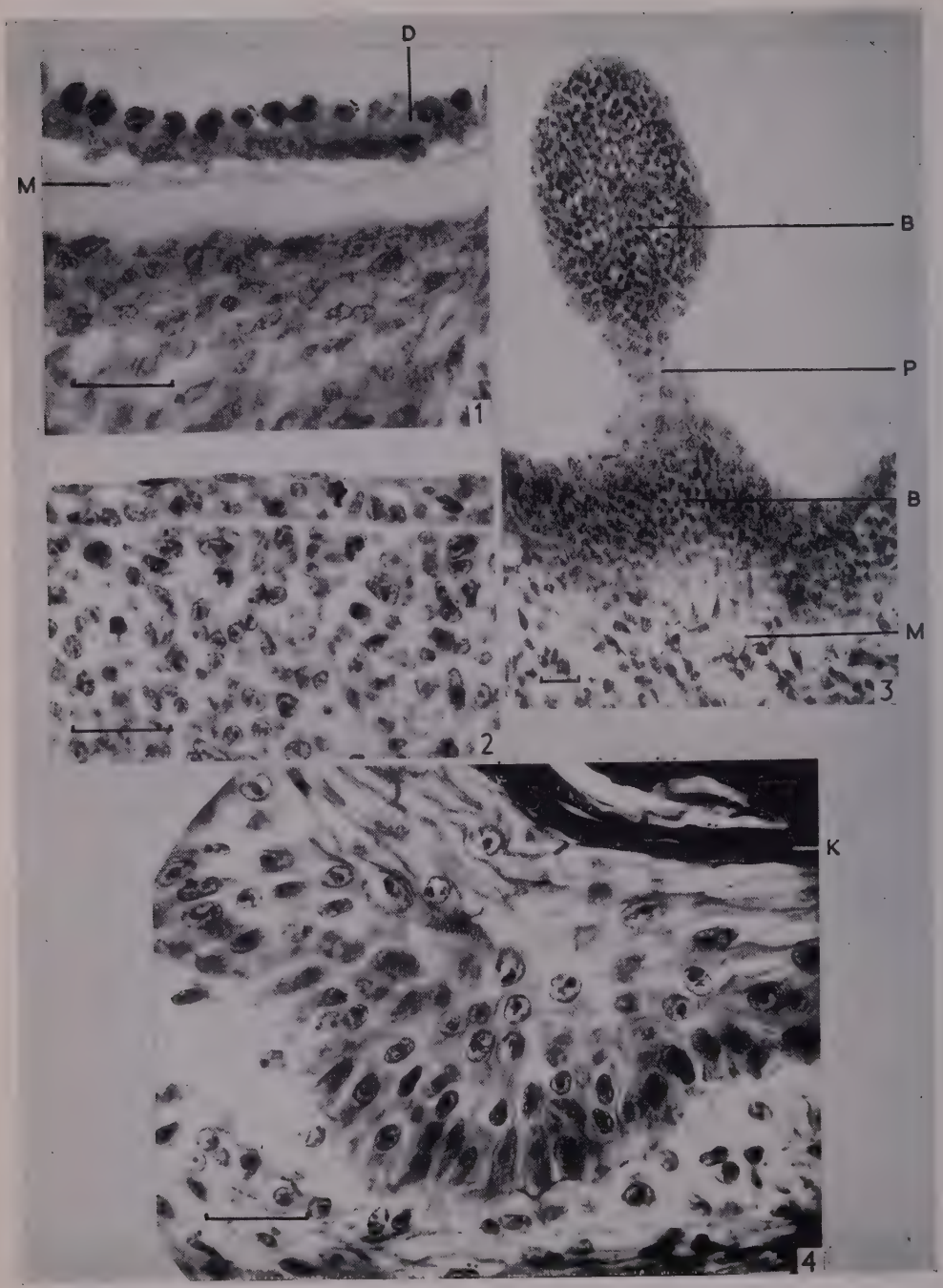

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Plate 1

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EXPLANATION OF PLATES

Scale line represents 20 μ throughout

PLATE 1

FIG. 1. Skin of a 5-day chick embryo fixed after 15 minutes' tryptic digestion. The epidermis is becoming detached, and draws with it a strand of PAS-positive intercellular material (*M*). Note the 2-layered structure of the epithelium, and the mitotic division (*D*). PAS. ×730.

FIG. 2. Section of skin from a 5-day embryonic chick. The epidermis is composed of an upper flattened peridermal layer, and a basal layer of stellate cells. Haematoxylin. ×750.

FIG. 3. Tangential section through the skin of a 5-day chick embryo showing the epithelial structure of the periderm (*P*); the basal layer of stellate cells is cut twice (*B*). Dermal mesenchyme (*M*) appears in the lower part of the picture. Haematoxylin and eosin. ×290.

FIG. 4. Section of explanted skin after 11 days *in vitro*. The epidermis is keratinizing normally (*K*), and contains intercellular bridges and tonofibrils in the lowerl ayers. Azan. ×730.

Plate 2

Figs. 5 *a* & *b*. Two areas from a whole-mount of an epidermal sheet growing *in vitro* on fibroblasts. The epidermal cells extend long processes containing spiral filaments of Herxheimer (*F*), and terminating in end-feet (*E*). Iron haematoxylin. ×750.

Fig. 6. Skin explant after 12 hours *in vitro*; the basal layer of the epidermis has become typically columnar, and the periderm is cuboidal with terminal bars (*T*). Note cell (*C*) already moving upwards from basal layer. Basement membrane not stained. Iron haematoxylin. ×750.

Fig. 7. Horizontal section through the epidermis of a skin explant grown for 8 days. The differentiated periderm is seen in surface view and shows irregular dark masses of trichohyalin (*Tr*) in the cells, and terminal bars (*T*) between them. Azan. ×730.

Fig. 8. Section through the isolated 5-day epidermis immediately after removal with trypsin. It retains its 2-layered structure. Haematoxylin and eosin. ×240.

Fig. 9. Section of isolated epidermis after 24 hours *in vitro*. Above, rounded peridermal cells (*P*) laden with phagocytosed cellular débris. Stellate 'basal' cells (*B*) on right are re-establishing contact through fine cytoplasmic extensions; elsewhere they are still scattered between numerous degenerating cells. Azan. ×670.

Plate 3

Fig. 10. Isolated epidermis after 6 days *in vitro*. The part of the explant on the right is completely keratinized, but on the left a core of living cells remains. Haematoxylin. ×180.

Fig. 11. Isolated epidermis is completely keratinized after 10 days *in vitro*. Note the small strand of PAS-positive intercellular material (*M*), derived from the mesenchyme, in the centre of this explant. PAS and haematoxylin. ×370.

Fig. 12. Culture of isolated epidermis after 6 days *in vitro*. It has enclosed a strand of cell-free mucoprotein derived from the intercellular material of the mesenchyme. The keratinizing epidermis is oriented around the mucoid (*M*). Azan. ×230.

Fig. 13. Culture of isolated epidermis explanted with cell-free intercellular material, after 12 hours *in vitro*. On the right, the cells in contact with the strand of mucoid (*M*) are oriented towards it and have assumed a columnar epithelial arrangement. On the left, by contrast, is a nodule of epidermis not associated with mucoid. Here, the cells are completely disoriented. Azan. ×330.

Fig. 14. Intercellular material acts as a basement membrane. Part of a culture of isolated epidermis explanted in contact with cell-free intercellular material, after 16 hours *in vitro*. The cells are oriented to the mucoid (*M*) as shown by their elongated nuclei and intracellular tonofibrils. Contrast with the lack of orientation in fig. 9. Peridermal cells are uppermost. Note the mitotic division (*D*). Azan. ×1050.

Fig. 15. Orientation spreads through an explant of isolated epidermis; 16 hours *in vitro*. On the left and below, already oriented cells are closely packed in an epithelial arrangement. On the right and above, the epidermal cells are still separated from each other, but are establishing contact by fine filaments (*F*). An orienting influence is proceeding diagonally from lower left to upper right. Iron haematoxylin. ×750.

Plate 4

Fig. 16. Explant of isolated epidermis in contact with cell-free intercellular material; 24 hours *in vitro*. Mucoid is in contact with the epidermis in the centre but not at the ends. The orienting influence of the strand of the mucoid (*M*) has now extended through many layers of cells: they have moved centrally towards it from both ends of the explant, and are heaped thickly above it. The surrounding peridermal cells (*P*) are conspicuous owing to their content of degenerating material. Azan. ×330.

Fig. 17. Nodule of isolated epidermis treated with excess vitamin A, 5 days *in vitro*. The cells show no sign of a squamous arrangement. Contrast with Plate 3, fig. 10. Haematoxylin and Alcian blue. ×300.

Fig. 18. Isolated epidermis treated with excess vitamin A, 6 days *in vitro*. Plentiful mucus secretion (*M*). Haematoxylin and Alcian blue. ×300.

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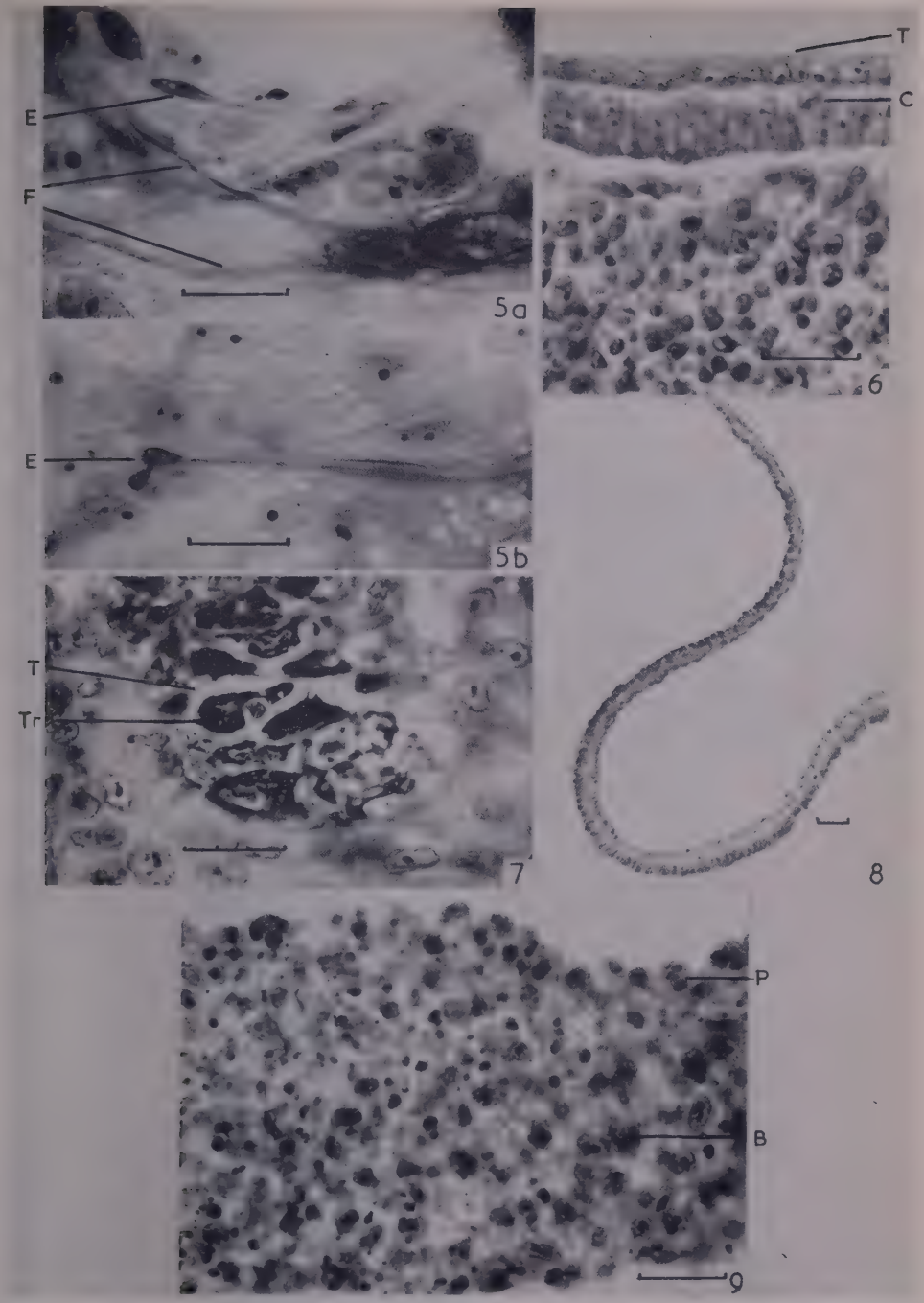

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Plate 2

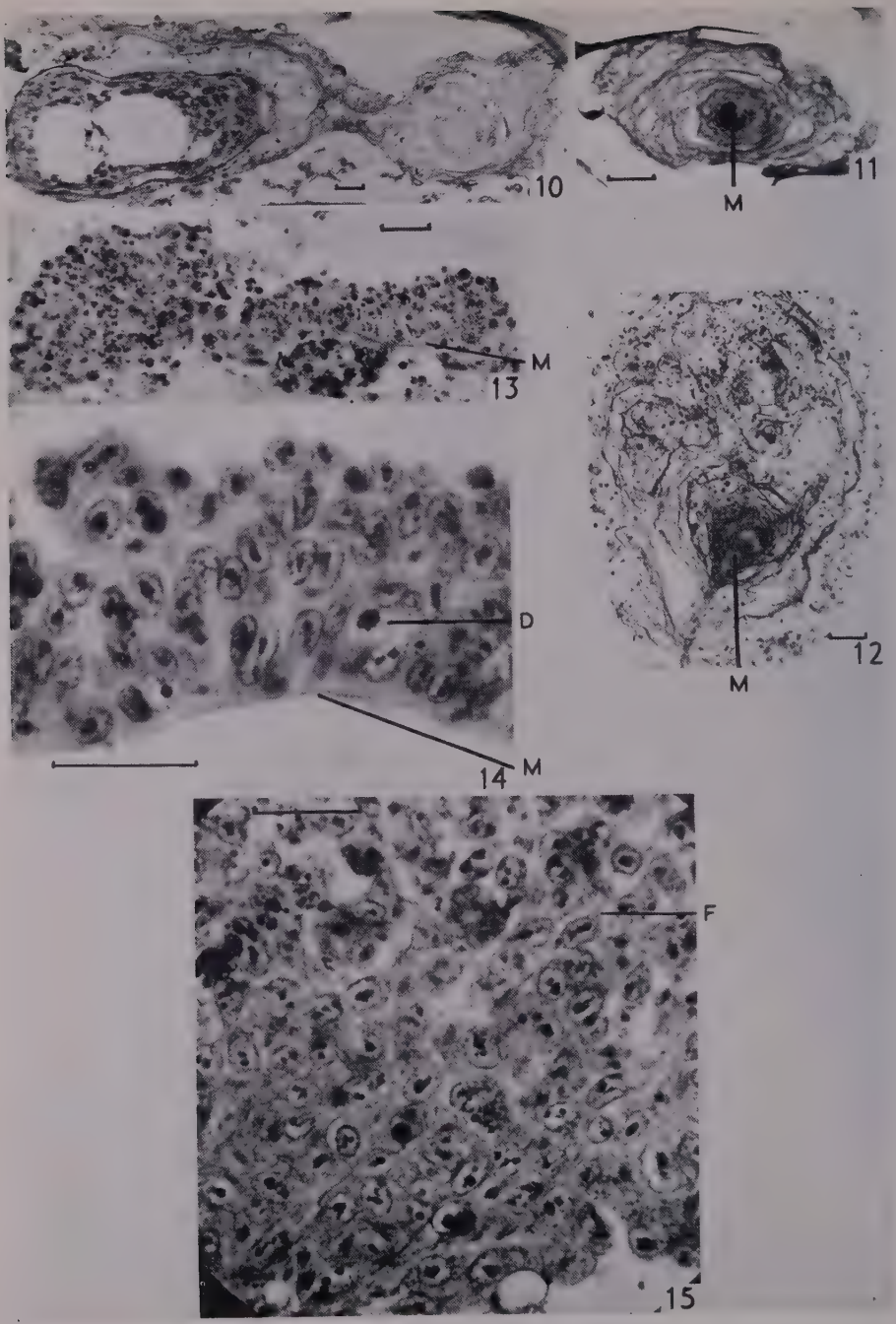

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Plate 3

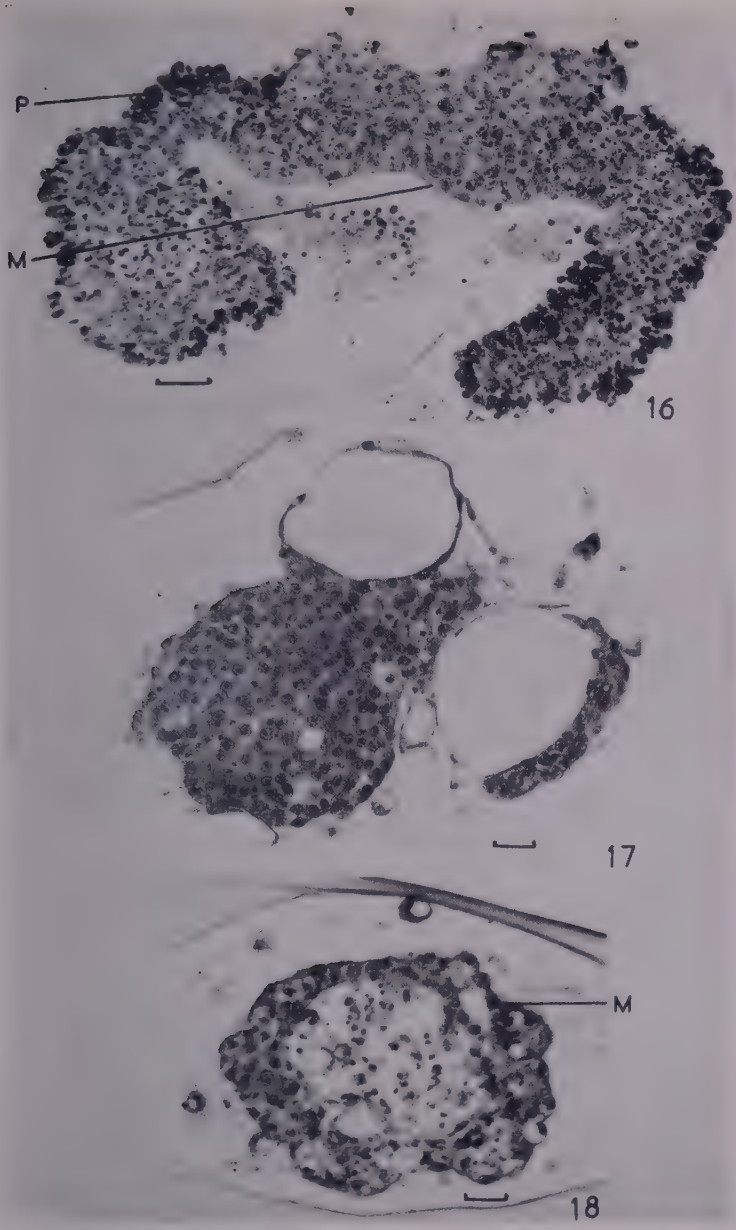

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Plate 4

The Importance of Mesenchymal Factors in the Differentiation of Chick Epidermis

II. Modification of Epidermal Differentiation by Contact with Different Types of Mesenchyme

by C. B. McLOUGHLIN[1]

From the Strangeways Research Laboratory, Cambridge

WITH FOUR PLATES

INTRODUCTION

THAT the mesenchyme may determine the differentiation of epithelial derivatives has been demonstrated in a wide range of embryonic organs, e.g. limb-bud (Zwilling, 1956; Saunders, Gasseling, & Gfeller, 1958), feather-germs (Sengel, 1956), preen gland (Gomot, 1958), salivary gland (Borghese, 1950; Grobstein, 1953), and thymus (Auerbach, 1960). These inductions probably act through various mechanisms, but a feature that is common to many of them is the relatively brief duration of the mesenchymal stimulus, e.g. the mesenchymal induction of feather-germs in the chick lasts for half a day only. This brevity of action is also characteristic of the classic inductions, e.g. of the neural plate or lens.

The work reported here (and briefly elsewhere, McLoughlin 1961*a*, *b*,) indicates that besides such brief inductions there are more prolonged epithelio-mesenchymal interactions which maintain the normal growth and differentiation of tissues throughout embryonic life, and possibly in adult life also.

The existence of such persistent influences is suggested by histological considerations (Kingsbury, Allen, & Rotheram, 1953; Sylvèn, 1950; Ozzello & Speer, 1958) and by various experimental data. For example, certain embryonic epithelia fail to grow when cultivated in the absence of mesenchyme, and it has been found (McLoughlin, 1961*a*) that the isolated epidermis from 5-day chick embryos keratinizes completely under these conditions. From these results it would seem that in the embryo the mesenchyme must exert a continuous action on certain epithelia, without which the epithelial cells cannot survive and multiply.

Sobel (1958) showed that the epithelium of the pituitary rudiment from an

[1] *Author's address*: Strangeways Research Laboratory, Wort's Causeway, Cambridge, U.K.

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8-day chick embryo can neither grow nor differentiate in the absence of connective tissue, but is able to do both when perichondrial fibroblasts from the epiphyses of embryonic long-bones are added to the culture; in this rudiment, therefore, epithelial growth and differentiation are controlled by contact with cells of a particular type, i.e. perichondrial fibroblasts, and it is immaterial whether these come from the normal site, i.e. the chondrocranium, or from the limb-bones. Auerbach (1960) separated the epithelium of the embryonic thymus (mouse) from its own mesenchyme and combined it with the mesenchyme of other organs, such as lung and submandibular gland, and concludes that 'three types of specificity seem indicated: 1. Mesenchyme derived from a variety of embryonic rudiments induces growth and morphogenesis in thymus epithelium. 2. The presence of thymic mesenchyme leads to immediate growth and morphogenesis whereas heterogeneous mesenchyme first causes rounding of the epithelium, morphogenesis and growth representing a distinct second phase of the response; and 3, the second phase of epithelial response to heterogeneous mesenchyme varies characteristically with the source of mesenchyme used.'

Thus it may be generally true that each type of mesenchyme has a characteristic influence on epithelia with which it is in contact. In order to investigate this question further, isolated epidermis was grown on various types of mesenchyme to see whether its differentiation would be modified by the cells with which it was combined. The results showed that each type of mesenchyme produced a characteristic effect on the implanted epidermis.

MATERIALS AND METHODS

Preparation of tissue

Usually 2 or 3 embryos were dissected in each experiment. The hind limb-buds, the stomach (consisting of proventriculus and gizzard), and the heart were removed and placed in Tyrode's solution with a few drops of embryo extract added (about 5 drops/ml.). The epidermis was removed from the hind limb-buds with trypsin as previously described (McLoughlin, 1961*a*), and transferred to 1:1 horse-serum and Tyrode's solution. It was marked with carbon particles so that later on in culture, it could be distinguished from any fragments of gastric epithelium which might accidentally have remained attached to the mesenchyme on which it was to be implanted. Soon after isolation each epidermal fragment curled up with the basal cells inwards. The limb mesenchyme from which the epidermis had been removed was placed in a separate dish containing a mixture of equal parts of horse-serum and Tyrode's solution.

Each stomach was divided into proventriculus and gizzard. These were treated separately with trypsin to remove the lining epithelium, which was usually discarded. The epithelium-free mesenchyme of each proventriculus was then usually divided into two, and that of each gizzard into three pieces, and these were also placed in horse-serum diluted 1:1 with Tyrode's solution. The

auricles were removed from the heart, and the ventricles cut into pieces corresponding in size with the fragments of gastric mesenchyme. Trypsin treatment is not necessary in the preparation of cardiac mesenchyme, but in some experiments the heart fragments were treated in the same way as limb and gastric mesenchyme in order to provide a control for any influence tryptic digestion might have on the mesenchymal factors that affect the implantation and differentiation of the epidermis; epidermis behaved in the same way on heart mesenchyme whether the heart had or had not been treated with trypsin.

Four groups of combined cultures were made, as shown in Table 1.

TABLE 1

Number of cultures of epidermis on different types of mesenchyme

Epidermis on mesenchyme from	*Number of cultures*
Limb (control series)	80
Gizzard	47
Proventriculus	37
Heart	48

Culture method

To prepare a combined culture, a fragment of epidermis and a piece of the appropriate mesenchyme were placed together in a drop of horse-serum Tyrode on a Maximow double coverslip. The epidermis, bearing a few carbon particles, was unrolled with fine, blunted glass needles, and floated basal side down over the mesenchymal fragment. The horse-serum/Tyrode was then withdrawn with a fine pipette so that the unrolled epidermis sank down and became draped over the mesenchyme, with which it established close contact. A drop of embryo extract was then mixed in a Petri dish with a drop of plasma, approximately in the proportion of 1 part of extract to 2 parts of plasma, and this medium was spread round the combined tissues. Both mesenchyme and epidermis are non-adhesive after treatment with trypsin, so it was difficult to add the permanent medium without disturbing the explant: the best way was to spread the medium in a ring round the combined tissues and allow it to converge simultaneously from all directions. In 1–2 minutes the plasma clotted and held the explanted tissues closely together. Each Maximow double coverslip was then sealed on to a depression slide and incubated at 38° C.

In preparing these cultures, it was essential to unroll the epidermis and place it with the basal layer in contact with the mesenchyme; if placed peridermal side down, the epithelium fails to become implanted, indicating an interesting difference in the capacities of the basal and peridermal cells even at this early stage. In the proventriculus and gizzard cultures the epidermis was usually placed in contact with the mucosal surface of the mesenchyme. Sometimes, however, this was not possible, and the epidermis became implanted on the cut or

serosal surface, but this did not affect the response of the epidermis to the mesenchyme.

The medium was replenished every 2nd day and the cultures were transplanted every 4th day, so that the tissues could retract and remain suitably thick for histological examination.

Histological methods

Explants were fixed daily, from 1 to 16 days *in vitro*, in Zenker's solution with 3 per cent. acetic acid or in Carnoy's mixture (ethanol 6 parts, chloroform 3 parts, acetic acid 1 part). While in absolute alcohol they were removed from the coverslip with a razor, then embedded in paraffin and serial sections 7 μ in thickness were cut at right angles to the coverslip. In addition to the usual staining methods such as Azan, iron haematoxylin, or Alcian blue (1 per cent. in distilled water, 10 seconds), the periodic-acid/Schiff technique (PAS) and basic dyes such as Toluidine blue (0·1 per cent.) and Azure A (0·01 per cent.) were used for the identification of mucopolysaccharides. Trevan's method (personal communication) was found particularly useful. This consists of an initial treatment with Alcian blue at pH 3 to pick out acid mucopolysaccharides, followed by PAS which stains the remaining neutral mucopolysaccharides red. Mercury orange was also used to stain specifically for disulphide and sulphydryl groups (Bennett & Watts, 1958).

RESULTS

Initial interaction of epidermis with mesenchyme

During the first 24 hours the reaction of isolated epidermis to the mesenchyme on which it is explanted is similar in all composite cultures (except epidermis on heart myoblasts). This reaction culminates in the establishment of a normal relationship between epidermal and mesenchyme cells, and in the re-establishment of an oriented epithelial structure in the epidermis. The following is a general description of this process and any individual variations will be described in the appropriate section.

The epidermis behaves for the first 24 hours as if it were isolated, i.e. it loses its 2-layered arrangement and rounds up into a disorganized nodule in which the living cells become separated from each other, and there are many degenerations (McLoughlin, 1961*a*). At the point where basal cells are in contact with the mesenchyme, however, they become oriented towards it in an orderly way to form a single columnar epithelial layer (Plate 1, fig. 2). The orientation is transmitted, through fine filaments by which the cells make contact with each other, to cells farther within the epithelial nodule, so that these in turn move towards the point of attachment and become arranged in several rows above the first established layer.

Some cells of the second epidermal layer, and even of the third and fourth, send long processes containing a thick tonofibril down between the cells of the

basal layer to make contact with the mesenchyme (Plate 1, fig. 3, *P*); sometimes this tonofibril is arranged in a fine spiral (Plate 1, fig. 4, *T*), and sometimes two or three cells send entwined processes which jointly penetrate the basal layer (Plate 1, fig. 3, *PP*). These cytoplasmic extensions resemble the spiral filaments of Herxheimer in normal skin: they end in minute branched feet, which give the impression of holding the cell anchored to the early basement membrane (Plate 1, fig. 5). The whole cell then seems to pull itself down by means of its process and squeezes in between the basal cells, so that it also becomes established on the basement membrane. To provide room for the incoming cells, the reconstructed epidermal sheet spreads gradually over the mesenchyme and the original epidermal nodule is thus depleted as its cells move downwards to make contact with the mesenchyme. This sequence of events often continues till the nodule disappears and a 2-layered epidermis has been reconstructed, after 48 hours *in vitro* (Plate 1, fig. 6).

At the point of contact with the epidermis, the mesenchyme soon forms a thin layer of mucopolysaccharide-containing, PAS-positive material, which represents a newly formed basement membrane. This becomes clearer as the epidermis spreads, so that, by the time it is fully established, a distinct basement membrane is present. The similarity between this initial reaction of epidermis to whole mesenchyme, and its response to isolated intercellular matrix of the mesenchyme (McLoughlin, 1961*a*,*c*), suggests that the reaction by which epidermis becomes established on the basement membrane is not primarily to the fibroblasts of the mesenchyme, but to the intercellular material they produce. The relation between epidermis and basement membrane described here recalls that observed in the amphibian tadpole in the electron microscope by Weiss (1958); there also the basement membrane is responsible for polarizing the epidermis, and the epidermis in turn organizes the basement membrane.

In all tissues except heart myoblasts, by the 4th day a distinct layer of fibroblasts can always be seen surrounding the epidermis, although the latter was initially implanted on tissue containing intermingled fibroblasts, myoblasts, and other cells. This indicates not only that epidermis and fibroblasts attract each other, but also suggests that the different and characteristic effects of the various mesenchymes on epidermal differentiation which will be described in the following sections, are the result of specific properties of the fibroblasts in the different mesenchymes.

The question arises of whether the newly formed basal layer and periderm in the reconstituted epithelium correspond to the original basal and peridermal layers. The following circumstantial evidence suggests identity of the original and reconstituted layers.

As stated above, the periderm will not become implanted on mesenchyme, so that epidermis will only 'take' if planted basal side down; it would be surprising, therefore, if peridermal cells take part in the formation of a new basal layer. Moreover, it has been noted (McLoughlin, 1961*b*) that the original peridermal

cells display an early phagocytic activity that is not shown by the basal cells. Since considerable degeneration occurs during the first 12 hours after the epidermis is explanted, the peridermal cells become distended with phagocytosed cellular debris and thus can be distinguished. In the 24-hour-old epidermal plaque, the cells that become oriented against the mesenchyme contain no visible degenerate material, while those that are left as an upper layer contain phagocytosed fragments, indicating that they probably represent the original peridermal elements. This view receives further support from the fact that the carbon particles added to the explanted epidermis as markers, are usually found in the peridermal cells of the reconstructed epidermis.

At 48 hours the basement membrane is very distinct, and the epidermis usually lies upon 3-4 layers of fibroblasts, which are forming collagen fibrils.

Epidermis on limb-bud mesenchyme

Isolated epidermis recombined with normal limb-bud mesenchyme forms a normal squamous keratinizing epithelium.

The normal limb-bud mesenchyme

In histological sections of the normal limb-bud of the 5-day chick, differentiation is fairly advanced in the most proximal region but diminishes in degree towards the tip where the cells are apparently undifferentiated. At all levels there is a central core of cartilage or procartilage cells surrounded by myoblasts, which in turn are enclosed by a layer of early fibroblasts.

Cultures

All regions of the limb-bud mesenchyme, except the most distal part, differentiate to produce a central cartilaginous nodule or group of nodules with radiating bundles of multinucleated embryonic muscle fibres containing striated myofibrils. Many fibroblasts mingle with the muscle fibres and spread more peripherally. The most distal region of the limb-bud usually gives fibroblasts only; a small cartilaginous nodule may also appear, but muscle fibres are absent.

Forty-eight hours after the explantation, together, of epidermis and mesenchyme, there is plentiful mesenchymal outgrowth, and the epithelium appears as a translucent plaque, indicating that a normal relationship has been established between the two tissues. After 3 days the epidermal plaque has usually extended a little; it rarely spreads farther after this stage, but usually rounds up to form a cyst, basal side outwards, and keratinizing inwards. The first signs of keratinization appear at about 5 days, and by 7 days the epidermis has usually formed a cyst lined with opaque keratin and surrounded by a condensed coat of dermal fibroblasts and their collagen fibrils (Plate 1, fig. 1). This appearance, with increasing deposition of keratin, is maintained throughout the culture

period. Often the epidermis that comes to lie on top of cartilage develops very poorly, and may actually degenerate. The dermal fibroblasts form many collagen fibrils coated with a mucopolysaccharide material that always stains red (for neutral mucopolysaccharides) with Trevan's method. In histological sections it can be seen that the newly formed dermis associated with the explanted epidermis thickens and the number of cells increases faster than in fibroblastic areas elsewhere in the culture (Plate 2, fig. 7). It is not known whether the epidermis achieves this by promoting mitosis in the dermis, or by trapping passing fibroblasts.

After 2 days *in vitro* the epidermis may be either a simple 2-layered epithelium, or an early stratified squamous epithelium. In most cultures it is beginning to roll up to form a cyst, and occasionally this process is already completed. From this point onwards most of the epidermal fragments differentiate normally, but those in contact with cartilage do not.

Four types of epidermal behaviour have been observed: normal keratinization, failure to thrive in contact with cartilage, cyst formation, and the secretion of fluid by newly formed cysts.

Normal keratinization. By the 3rd to 5th day the epidermis has already become a stratified squamous epithelium: between the 5th and 7th days keratin begins to appear and the stratum corneum grows progressively thicker. The differentiation of epidermis recombined with limb mesenchyme (see Table 2) is usually indistinguishable from that of undisturbed epidermis, and, indeed, sections of these explants illustrate the normal process of keratinization very clearly (Plate 2, fig. 7).

Epidermis on cartilage. Though the epidermis initially becomes implanted normally on perichondrial fibroblasts, it develops very poorly. The basal layer becomes disorganized, and the epithelium tends to spread; mitoses are rare, and differentiation is slow. Finally, the epidermis either degenerates completely or else keratinizes down to and including the basal layer. In early stages (up to 4 days), the cartilage often seems to suffer from the proximity of the epidermis; less matrix is deposited than elsewhere and many chondroblasts may become filled with iron-haematoxylin-staining droplets and degenerate. Some normally keratizing epidermal cysts lie with one end in contact with the perichondrium of a cartilaginous nodule, and this small region differentiates abnormally (Plate 2, fig. 8). This localized response to the perichondrium on the part of an otherwise normal cyst provides a particularly clear illustration of the incompatibility of the two tissues. It is suggested that cartilage is not capable of providing a normal basement-membrane for the epidermis. Of a total of 80 control cultures, 66 differentiated normally, while 14 came to lie on cartilage and with which they were incompatible (see Table 2).

Cysts. These may be formed in young cultures in one of two ways. An entire epidermal plaque may early become enclosed by fibroblasts so that a basal layer is formed all round the epidermal nodule. A central space forms in the

plaque by the extreme vacuolar distension of a single cell (such distension is common in dying 'disoriented' epidermal cells). This space becomes surrounded by flattened peridermal elements applied to the thin cytoplasmic wall of the distended cell, and any remaining epidermal cells become oriented between the central lumen and the surrounding basal layer to form a stratified epithelium. The cytoplasm of the inmost distended cell finally becomes indistinguishable from the distal surfaces of the peridermal cells.

Alternatively, a cyst may be formed from an open epidermal sheet when the initial spreading movement has been halted. Dermal fibroblasts migrate over the edge of the sheet and move on to the surface of the epidermis, drawing the edges of the sheet with them: this causes a general inrolling around the edges of the epidermis. Fibroblasts on top of the sheet converge from all sides towards the centre, in much the same way in which a bag is closed by pulling a draw-string; the epidermis is pulled in their wake, and finally the inrolled margins fuse to form the upper side of an epithelial cyst which keratinizes inwards. Usually both these processes occur simultaneously in the same culture, so that several cysts are formed which may later fuse.

TABLE 2

Differentiation of epidermis recombined with limb mesenchyme

Age (days)	*No. of cultures*	*Undifferentiated*	*Stratified squamous*	*Keratinized*	*Degenerate*
		I. *On dermal fibroblasts*			
0–5	31	15	12	4	—
6–11	25	—	13	12	—
12–16	10	—	—	10	—
		II. *On cartilage*			
0–5	5	4	1	—	—
6–11	6	1	3	—	2
12–16	3	—	—	1	2
	80	20	29	27	4

Secretion of fluid. When cysts are formed very early, i.e. about the 2nd day *in vitro*, the epidermis is still in a simple 2-layered state and appears to be able to produce fluid as long as it remains so. These cysts become distended with fluid, and consequently the epidermis preserves its primitive 2-layered structure for an unusually long time. A total of 12 such cysts were noted: no trace of mucus has ever been seen in them, and after about 5 days very flattened squamous layers always begin to appear. As keratinization begins (Plate 2, fig. 8) fluid is reabsorbed, and the once distended vesicles shrink and become filled with keratin. This observation that the simple 2-layered epidermis secretes fluid, suggests that it may be responsible for forming amniotic fluid in the chick embryo. The differentiation of epidermis on limb mesenchyme is summarized in Table 2.

Epidermis on gizzard mesenchyme

Epidermis implanted on gizzard mesenchyme fails to keratinize, but forms mucus and may become ciliated.

The normal gizzard

At 5 days this is a thick-walled organ lined with a pseudostratified columnar epithelium which secretes mucus that stains blue for acid mucopolysaccharides with Trevan's method. The mesenchyme contains fibroblasts whose matrix stains slightly for acid mucopolysaccharides, numerous young smooth myoblasts, and scattered intramural ganglia with tracts of unmyelinated nerve fibres accompanied by Schwann cells. Surrounding the whole organ is the serosal lining which is usually underlain by a thin layer of fibroblasts.

Many simple glands arise in the lining of the gizzard. At 8 days their secretion stains blue for acid mucopolysaccharides with Trevan's method, but at 17 days it is undergoing transformation to the adult horny lining, which is described by Aitken (1958). This secretion is produced as a fluid, but as it moves into the lumen of the gizzard it condenses to the characteristic horny material, which is resistant to digestion with hot 5 per cent. KOH. Near the secretory cells it stains for acid mucopolysaccharides, but farther out in the lumen it stains for neutral mucopolysaccharides only. Mercury orange staining shows that it is very rich in SH and S—S groups, so perhaps it hardens by the oxidation of sulphydryl groups to disulphide bonds.

There seem to be fewer fibroblasts per area in gastric than in dermal mesenchyme and the connective tissue they form contains finer bundles of collagen fibrils that lie in a more abundant matrix, which stains faintly for acid mucopolysaccharides. The myoblasts differentiate into elongated smooth muscle fibres, and are interwoven into a powerful muscular mass. Small autonomic ganglia interconnected by tracts of unmyelinated nerve fibres are scattered among these muscle fibres.

Cultures

Five-day gizzard mesenchyme explanted *in vitro* differentiates into groups of smooth muscle fibres interlaced with fibroblasts and penetrated by ramifying bundles of unmyelinated nerve fibres connecting numerous small autonomic ganglia. Vascular spaces are present. Peristalsis is occasionally observed between the 3rd and 6th day *in vitro*.

Epidermis explanted together with gizzard mesenchyme has usually become completely fused with the latter by the 2nd day, and can often be distinguished as a clear plaque. In contrast with its behaviour on limb mesenchyme, where it rolls up to form cysts, the epidermis shows a tendency to spread uncontrollably on gizzard mesenchyme: this may continue for up to 8 days, so that the epidermis often forms a hillock inside which the mesenchyme collects. As the epidermis

continues to envelop the mesenchyme, this hillock gradually becomes pedunculate, finally detaches itself from the rest of the culture on the cover-glass, and may float freely as an isolated sphere of mesenchyme covered with epidermis. This tendency for the epithelium to extend its area of contact with the mesenchyme is characteristic of explants from any part of the digestive tract (Walker, personal communication). Thus epidermis on gizzard mesenchyme strikingly resembles gastric epithelium in this respect.

This behaviour, though interesting in itself, caused technical difficulties since cultures enveloped by epithelium provide poor material for observations on the differentiation of the epidermis. In later experiments, therefore, efforts were made to promote the formation of cysts by placing the isolated epidermis in a groove in the mesenchyme at the time of explantation. Under these conditions the epidermis forms a plaque surrounded by mesenchyme, and by the 3rd day a cyst usually develops by cavitation within the solid plaque, as described for epidermis on limb mesenchyme.

On the following days the cyst secretes fluid with which it is increasingly distended; its outline becomes refractile, which was found to indicate mucus secretion, and individual cells are seen to contain droplets of refractile material (Plate 2, fig. 9). Serial daily photographs show that the amount of fluid contained in such a cyst continues to increase until about the 9th day in culture. Occasionally these cysts collapse before the end of the culture period, owing to their extreme dilation.

Histological observations show that during the first 24 hours, epidermis explanted on gizzard mesenchyme establishes itself in the manner described in the first section. It responds more rapidly to gizzard than to limb mesenchyme, so that by the end of the 1st day more cells are seated on the basement membrane than in the limb-bud cultures, and by the 2nd day a simple 2-layered epithelium has always been reconstructed. This consists of a cubical basal layer covered with a cubical peridermal layer which contains most of the marking carbon particles. Occasionally a little mucus, staining with Alcian blue, is already present on the epithelial surface, and by the 3rd day this is always unmistakable as a distinct but very thin line.

As on limb mesenchyme, the epidermis is always found to lie upon fibroblasts. These form a basement membrane within the first 2 days, and this stains as a neutral mucopolysaccharide with Trevan's method, but the intercellular material and matrix surrounding the collagen fibrils in the mesenchyme always stains partly or entirely as an acid mucopolysaccharide.

The differentiation of epidermis in cysts will be described first, since it is the most frequent type of development. As stated above, epidermis on gizzard mesenchyme produces much fluid, and one advantage of the arrangement of the epidermis in cysts is that the fluid is collected so that its amount can be assessed visually. The production of fluid usually begins on about the 3rd day, and observations of living cultures show that it may continue to increase until

about the 9th day: fluid production appears to continue as long as the epidermis remains a simple 2-layered epithelium.

During the first 6 days the epidermis is arranged in a double columnar layer (Plate 2, fig. 10). The peridermal cells show distinct terminal bars and produce small quantities of mucus, but do not form typical goblet cells: the basal cells assume a tall columnar arrangement. A typical single-layered columnar epithelium is never formed, and the cells remain recognizably epidermal although their differentiation is much modified.

Basal cells on gizzard mesenchyme have a swollen nucleus with a very large nucleolus and clear cytoplasm with relatively few thin tonofibrils; thus they contrast with the basal cells of normal epidermis, which have smaller nuclei, basophilic cytoplasm, and abundant tonofibrils. Spiral filaments of Herxheimer are absent from epidermis on gizzard; intercellular bridges occur, but the cells are usually in such close contact that the bridges remain inconspicuous. The differences from the normal in the content and arrangement of tonofibrils are best seen in whole mounts of epithelial sheets. After 8 days a few layers of cells may be produced between the basal layer and the periderm; these may assume a squamous shape (Plate 2, fig. 11), but never show any sign of keratinization. Mucus secretion begins on about the 3rd day and increases slowly to about the 7th day (Plate 2, fig. 11). Cilia are occasionally observed on peridermal cells only, in scattered areas of the cyst wall.

Between the 12th and 14th day these distended cysts often collapse. Mitoses are usually not seen after about the 9th day, and subsequently part of a cyst may become eroded owing to degeneration. The changes in the thickly heaped-up epidermis of such a collapsed cyst are of interest.

TABLE 3

Differentiation of epidermis implanted on gizzard mesenchyme

Age (days)	*Number of cultures*	*Mucus*	*Cilia*	*Stratified squamous*	*Keratin*
1–2	3	1	1	—	—
3–6	14	13	5	1	—
7–10	15	13	5	2	1
11–14	15*	10	—	—	3
	47	37	11	3	4

* In this group two epidermal cysts degenerated.

Immediately after the collapse, the peridermal cells which have been carried into the centre of the cyst form a peculiar secretion, sometimes in large quantities. This secretion may stain blue with Trevan's method, where it lies in contact with the cells, but red for neutral mucopolysaccharides in the lumen of the cyst. It shrinks on fixation and stains strongly with Mercury orange for SH and S—S groups. In all these respects it resembles the normal secretion of the

gizzard, but the meaning of this resemblance is doubtful, since a similar material is produced under some circumstances by epidermis on proventriculus and heart, and rarely even on limb mesenchyme (McLoughlin, 1961*b*). It seems to be formed instead of trichohyalin, the characteristic intracellular product of peridermal differentiation, when the epidermis keratinizes very rapidly beneath the periderm.

Many epidermal cells become heaped together in the centre of the cyst below the peridermal cells. Some of these, which are far removed from contact with the mesenchyme, follow their normal fate and form keratin. (In Table 3 the four explants in which keratin appeared were of this type.) This indicates that the gizzard mesenchyme exerts a continuous influence on epidermal differentiation, but that the influence extends only over a limited range; its effects are reversible, for when it is removed the epidermis can keratinize.

In these collapsed cysts, the cells of basal origin, which no longer have a peridermal covering, often form characteristic goblet cells (Plate 2, fig. 12). From this it is clear that, although mucus secretion in these cultures is usually performed by the periderm as the most distal layer of the epithelium, the basal cells are sufficiently transformed to secrete mucus themselves. After about 11 days, some of the epidermal cysts begin to degenerate.

Though sheets occur less often than cysts (12 sheets to 35 cysts) they were sufficiently numerous to provide a parallel with each stage of differentiation of the cystic epidermis. The comparison is valuable since in open sheets the factor of distension with fluid and the subsequent tension, which might affect the differentiation of the epithelium, is eliminated. The differentiation of epidermal sheets on gizzard does not differ in any essential way from that in cysts. Ciliation of the peridermal layer (Plate 2, fig. 13) was found more often in open sheets than in cysts, i.e. in 8 out of 12 sheets as compared with 3 out of 35 cysts. The epithelium may become stratified, but usually shows no sign of a squamous structure and never forms keratin (Plate 2, fig. 13).

The differentiation of epidermis on gizzard mesenchyme is summarized in Table 3. It is to be noted that some cultures both secreted mucus and bore cilia. Thus gizzard mesenchyme influences epidermis to approach the form of a cuboidal epithelium, to secrete mucus, and to develop cilia on the peridermal layer.

The possible role of vitamin A in the response of epidermis to gizzard mesenchyme

The similarity between the responses of epidermis to excess vitamin A (Fell & Mellanby, 1953), and to gizzard mesenchyme suggest a common cause, e.g. the gizzard might store vitamin A and thus influence the epithelium to secrete mucus. Dr. Moore of the Dunn Nutritional Laboratory, Cambridge, kindly assayed stomachs from 5- and 13-day embryos by the method of Carr & Price (1926) for their vitamin A content. No vitamin was found, nor could any be

seen in frozen sections under the ultra-violet microscope; this excludes the presence of a very high concentration in the stomach. Although a permissive amount of vitamin A is probably necessary for the epidermis to secrete mucus in response to gizzard, a high concentration is unlikely to be a primary cause of this response.

Gizzard epithelium on limb mesenchyme

In order to see whether dermal fibroblasts would have the converse effect on gizzard epithelium, six fragments of gizzard epithelium were explanted on to limb mesenchyme. The gastric epithelium became established and surrounded by a thick coat of dermal fibroblasts, but its differentiation remained unaltered; it persisted as a columnar, mucus-secreting epithelium (Plate 3, fig. 14). Instead of spreading uncontrollably, however, as it does on its own gizzard mesenchyme, on limb mesenchyme it became rounded up to form compact cysts which produced their secretion inwards. Thus we have the following set of paired observations:

{Epidermis on limb mesenchyme forms cysts.
{Gizzard epithelium on limb mesenchyme forms cysts.

{Epidermis on gizzard mesenchyme spreads.
{Gizzard epithelium on gizzard mesenchyme spreads.

From this it was concluded that the spreading behaviour of an epithelium on a mesenchyme is not primarily a property of the epithelium, but may be determined by the mesenchyme.

Epidermis on proventriculus mesenchyme

In contact with proventriculus mesenchyme, epidermis initially reacts in the same way as it does to gizzard mesenchyme, i.e. by becoming a mucus-secreting epithelium; but after about 7 days it reverts to its characteristic differentiation and keratinizes normally and abundantly.

The normal proventriculus

This is a tubular dilation of the gastric end of the oesophagus, and is demarcated from the gizzard by a slight constriction. In histological detail it closely resembles the gizzard; the matrix laid down by the fibroblasts stains lightly blue with Trevan's method.

At 7 days, elevated rings with a central depression begin to appear in the proventricular lining, and at 8 days the depressions have already deepened to form tubular diverticula into the mesenchymatous wall of the organ. By 17 days the wall of the proventriculus contains a mass of compound diverticula whose alveoli are lined by simple tubular glands which produce a material rich in SH and S—S groups that resembles the secretion of the gizzard.

Cultures

The differentiation of proventriculus mesenchyme *in vitro* is indistinguishable from that of gizzard mesenchyme. As with epidermis on gizzard mesenchyme, efforts were made to promote the formation of cysts.

The behaviour of epidermis on proventriculus mesenchyme resembles that on gizzard mesenchyme for the first 4 days, i.e. thin-walled cysts are formed with a refractile lining indicating mucus secretion (Plate 3, fig. 15*a*). After 4 days these cysts are seen to accumulate less fluid than cysts of epidermis on gizzard mesenchyme, and by the 7th day they usually begin to keratinize and simultaneously lose what fluid they contain (Plate 3, fig. 15*b*). Keratinization usually begins in the region of the epidermis that is farthest from the centre of the culture, and progresses rapidly from the 7th day onwards.

The histological study of cultures fixed from day 1 to day 14 confirms that epidermis on proventriculus mesenchyme initially behaves in every detail as it does on gizzard mesenchyme; the construction of a 2-layered epithelium which immediately begins to secrete small quantities of mucus (Plate 3, fig. 16) and the continued production of mucus up to about 6 days are identical in the two types of composite cultures, but with the proventriculus the production of fluid and mucus stops at about 6 days. The epidermis then becomes stratified and squamous (Plate 3, fig. 17), the fluid in the cyst is reabsorbed or lost, and normal keratinization proceeds very rapidly (Plate 3, fig. 18); in some older cultures it even involves the basal layer so that no living epidermal cells remain. The keratin carries the peridermal lining of the young cyst, as a row of cubical cells, into the centre of the cyst. As the epidermis thickens below them, the peridermal cells often produce an abundant secretion of the neutral mucopolysaccharide-containing material rich in SH and S—S groups that is formed by peridermal cells under similar circumstances on limb and gizzard mesenchyme. Soon after keratin appears, these cells degenerate.

TABLE 4

Epidermis on proventriculus mesenchyme

Age (days)	*Number of cultures*	*Mucus*	*No mucus No keratin*	*Keratin*
1	1	—	—	—
2–4	4	4	—	—
5–6	7	6	1	—
7–8	10	3	2	5
9–14	15	—	2	13
	37	13	5	18

The results of explantation of epidermis on proventriculus mesenchyme are summarized in Table 4, from which the transition from a mucus-secreting to a keratinizing epithelium at about 7 days emerges clearly. All the explants

listed in which keratin was formed, contained a central group of cuboidal peridermal cells together with traces of mucus, as an indication of their history (see Plate 3, fig. 15).

These results suggest that the proventricular mesenchyme may contain the same factor as the gizzard mesenchyme, which influences the epidermis to differentiate into a cuboidal, mucus-secreting epithelium, but that it is present in a smaller quantity than in the gizzard. To investigate this possibility, the differentiation of six epidermal fragments each on an entire proventriculus was compared with the differentiation of six similar fragments each on a very small portion of gizzard. All six cultures of epidermis on proventriculus secreted mucus initially, halted, and then keratinized, whereas all the explants of epidermis on gizzard failed to keratinize and continued to form mucus. Thus the epidermis appears to respond to differences between the cells of the two organs, rather than to differences in the absolute amount of a stored active substance.

Epidermis on heart mesenchyme

Epidermis in contact with heart mesenchyme spreads to a single-layered epithelium on myoblasts, but rapidly keratinizes on fibroblasts.

The normal chick heart

The heart of a 5-day chick embryo is a well-differentiated, actively functioning organ. The ventricles consist predominantly of a spongy meshwork of muscle-cells with a layer of young myoblasts immediately within the epicardium; the folded endocardial surface is lined by flattened endothelial cells. In the valvular and septal regions of the ventricle there is a mass of fibroblasts, with an abundant matrix rich in acid mucopolysaccharides.

Cultures

Explants of chick heart produce a plentiful outgrowth within the first 2 days, and the culture becomes subdivided into two regions—a central area consisting mainly of myoblasts which continue to beat for 10 to 11 days, and a peripheral region containing most of the fibroblasts. Epidermis on heart mesenchyme behaves differently in contact with these two regions.

Epidermis which establishes contact with the myoblastic region is detectable as a dark nodule on the 1st day of culture, but by the 2nd day it has always spread widely and become translucent; by the 3rd day it is so thin that it is no longer distinguishable. When the cultures are cut out to be transplanted on the 4th day, retraction of the explant often reveals the formation of an extremely thin-walled fluid-filled cyst, which was previously so extended that it was undetectable (Plate 4, fig. 19). Where a strand of epidermis runs across the centre of a cyst, out of contact with the myoblasts (Plate 4, fig. 19), it thickens and begins to keratinize normally. Such cysts may continue unchanged throughout the culture period.

When the epidermis becomes implanted in the peripheral fibroblastic region,

it seems never to spread beyond its first point of attachment, which nearly always remains small. On the 2nd day it thickens, and begins to keratinize. The majority of the epidermal explants come to lie partly on the myoblastic and partly on the fibroblastic region, and differentiate accordingly. Occasionally, an epidermal explant which initially appears to be developing as a thin-walled cyst in the myoblastic region, draws together and forms a thick nodule of keratin, apparently surrounded by myoblasts; histological sections show, however, that the keratinizing epidermis is surrounded by a thin layer of fibroblasts which have migrated in between the myoblasts and the epidermal lining of the cyst.

The two regions of the heart explants can easily be distinguished in histological sections. With Azan and Trevan's method, most of the collagen is found in the peripheral zone of outgrowth, indicating the presence of the majority of the fibroblasts; only a few remain in the myoblastic area. The matrix formed by heart fibroblasts *in vitro* stains from mauve to blue with Trevan's method, and is strongly metachromatic with Azure A, i.e. it contains acid mucopolysaccharides.

From the moment of contact, epidermis on heart mesenchyme reacts in very different ways to the two types of cells with which it is confronted. (1) Heart fibroblasts strongly inhibit spreading of the epidermis, which consequently does not reconstruct to form a 2-layered epithelium but acquires a stratified squamous structure by the 2nd day. As the epidermal cells keratinize the periderm frequently secretes a large quantity of neutral mucoprotein material. The basement membrane stains for neutral mucopolysaccharides with Trevan's method, although the matrix produced by the fibroblasts generally contains a preponderance of acid mucopolysaccharides. Tangential sections show that tonofibrils are more numerous and closely packed than in epidermis on limb mesenchyme and the keratin formed is correspondingly more compact (Plate 4, fig. 20). After about 11 days the epidermis often becomes completely keratinized, down to and including the basal layer. (2) On myoblasts the epidermis spreads from the beginning as a single sheet of flattened cells which glide swiftly over the myoblastic region and may cover it entirely by 48 hours. When the spreading epidermal cells make contact with the surrounding fibroblasts, their progress is immediately halted. They pile up to form a stratified squamous epithelium around the myoblastic fibroblastic boundary, and peridermal cells and cells of basal origin once more become distinguishable (Plate 4, fig. 21).

Sometimes the epidermis becomes completely surrounded by myoblasts and develops into a clear fluid-filled cyst, but more often the epidermal cells are too numerous to be all accommodated in a single squamous layer on top of the myoblastic region, and spread until some pile up on the fibroblastic zone. Thus epidermis on heart mesenchyme often lies partly on myoblasts and partly on fibroblasts.

Epidermal cells in contact with heart myoblasts retain their squamous shape

throughout the culture period of 16 days (Plate 4, figs. 22 *a*, *b*). The basement membrane, if one is present, has become too thin to be seen. This single-layered squamous epithelium is so flattened that it resembles the endothelial lining of the heart, but it is known to be of epidermal, not endothelial, origin for the following reasons: (i) no similar cysts or sheets are found in cultures of heart mesenchyme alone; (ii) such sheets have been observed emerging from implanted epidermal fragments on the 1st day in culture; (iii) the single-layered epithelium sometimes carries carbon markings; (iv) where a cyst lies partly in the myoblastic and partly in the fibroblastic region, the cells in contact with the myoblasts remain flattened throughout the culture period, but an abrupt transition to a stratified squamous keratinizing epithelium occurs at the boundary of the myoblastic and fibroblastic regions; and (v) when a thin-walled cyst collapses and its epithelial lining becomes piled up, keratin is formed.

The question arises of whether the beating of heart myoblasts affects the differentiation of epidermis on heart mesenchyme. To answer this question, the beating was inhibited in 16 cultures by growing them in medium in which the proportion of embryo extract to plasma was 1 : 1, instead of the standard proportions of 1 : 2. The beating of heart myoblasts was inhibited from the 2nd day. This inhibition is probably due to the elevated level of potassium in the medium, since embryo extract is enriched by the intracellular K^+ of broken embryonic cells. Histological sections showed that up to about the 8th day the epidermis in these cultures differentiated exactly as in those that had not been prevented from beating; thereafter the immobilized muscle fibres began to degenerate, and fibroblasts migrated between them and the epidermis which simultaneously became transformed from a single squamous layer to a stratified squamous, keratinizing epithelium. The characteristic responses of epidermis to heart myoblasts and fibroblasts are therefore not the result of the regular beating of the myoblasts in culture, but are direct responses to the different properties of the two types of mesenchyme cells.

Table 5 summarizes the differentiation of epidermis on heart mesenchyme.

TABLE 5

Differentiation of epidermis on heart mesenchyme

Implanted on	*Number of cultures*	*Keratin*	*Single squamous*	*Half keratin Half single*
Fibroblasts	19	19	—	—
Myoblasts	8	—	8	—
Both	21	—	—	21

DISCUSSION

Since epidermis keratinizes in isolation (McLoughlin, 1961*a*) it is clear that contact with the various types of heterotypic mesenchyme, whose effects have been described, diverted the epithelium from its determined fate.

The response of epidermis to gizzard mesenchyme closely resembles its reaction to excess vitamin A, but no connexion has been found between the two stimuli. That such similar responses follow upon two apparently unrelated stimuli suggests that the epidermis has a limited repertory of forms of differentiation, and that its reaction to any of a wide range of stimuli is restricted to one or other of the various possibilities open to it. Except on heart myoblasts, the epithelium remains recognizably epidermal, e.g. intercellular bridges and tonofibrils persist, though they may be much modified. It is interesting that several types of mesenchyme evoke an appropriate response in the implant, i.e. each modifies the differentiation of the epidermis so that it comes to resemble the epithelium that normally clothes that mesenchyme. For instance, gizzard mesenchyme influences epidermis to resemble, as far as possible, the early, mucus-secreting gizzard epithelium; proventriculus mesenchyme has a similar, but weaker effect; and finally, heart myoblasts induce the epidermis to spread almost as thinly as the normal endocardial lining. An exception to this generalization is provided by heart fibroblasts, which are not normally in relation with any epithelium but cause epidermis to keratinize even more heavily than usual. Epidermis cannot establish itself on cartilage and withdraws from chondrifying tissues as does capillary endothelium (Clark & Clark, 1942).

An appropriate influence of mesenchyme on an epithelium was also found by Moscona (1961) in combined cultures of epidermis on mesenchyme from the oviduct of the 19-day chick embryo. When the explants were treated with oestradiole benzoate, the epidermis was transformed into a typical columnar mucus-secreting epithelium. Isolated epidermis treated with the oestrogen did not secrete mucus, so this effect seems to be mediated through the mesenchyme.

The range of mesenchymal tissues that have been found to exert characteristic effects on epidermal differentiation, permits the generalization that in mesenchyme cells specific factors are permanently present which can influence the differentiation of neighbouring epithelia, and which may be important in embryonic development. The interest of the results reported here is thought to lie less in the exact nature of the epidermal response than in the detection, through the epidermal response, of these normal factors in the mesenchyme.

Several observations suggest that the influence of the mesenchyme on the epithelium is reversible, e.g. when a cyst of epidermis in gizzard or heart myoblasts collapses and the epithelium piles up thickly so that it is spatially somewhat removed from the mesenchyme, it keratinizes; also, proventriculus influences the epidermis first in one way and then in another. This indicates that the mesenchymal influences discussed here must act continuously in order to maintain the modified differentiation of the epidermis. Such continuity of the mesenchymal action, and the reversibility of its effect, contrasts with the classical concept that 'normally, of course, the tissues cease to be plastic as soon as they have undergone the inductive action of an organiser' (Huxley & de Beer, 1934). Although the individual tissue interactions described in this paper clearly play

no part in normal development (they occur between types of tissue that normally are never in contact), they demonstrate two important histogenetic principles: firstly, that mesenchymal factors are probably of widespread significance in influencing and maintaining the differentiation of overlying epithelia; and secondly, that, since the differentiation of the early embryonic epidermis of the chick is susceptible to modification by such factors, the maintenance as well as the appearance of epidermal derivatives may depend on the underlying mesenchyme. The interesting possibility is also raised that wherever cells of different types come into contact they may always influence each others' differentiation.

Mesenchyme can influence not only the differentiation but also the growth of overlying epithelia. In the preceding paper (McLoughlin, 1961*a*) it was noted that many embryonic epithelia are unable to undergo mitosis when cultured in the absence of mesenchyme cells, i.e. that contact with mesenchyme is of general and basic importance to developing epithelia. When epidermis is replaced on limb mesenchyme a normally dividing basal layer is re-established and persists throughout the culture period. By contrast, on cartilage, heart fibroblasts and proventriculus, the ability to divide is restored to the epithelium at first but gradually the mitotic rate declines, and towards the end of the culture period the epidermis sometimes keratinizes down to and including the basal layer. This failure of several heterotypic mesenchymes to support mitosis consistently, indicates that only the dermal influence is correctly adapted to the epidermis. Similarly, Drew (1923) found that when he combined epithelia and fibroblasts of different origins in culture one of the tissues usually failed to survive.

The characteristic effects that each type of mesenchyme exerts on the epidermis are thought to be exercised particularly by the fibroblasts, since these congregate specifically around the epithelium in all composite cultures (except in epidermis on heart myoblasts). It is clear from existing data that fibroblasts and their products differ sufficiently in various tissues to provide a possible basis for the morphogenic effects described. In dermal mesenchyme *in vitro*, collagen fibrils coarse and coated with neutral mucopolysaccharide are abundant, while proventriculus, gizzard, and heart mesenchyme contain fine collagen fibrils which often lie in a matrix rich in acid mucopolysaccharides. It is well known that in the adult animal the connective tissues of different regions exhibit variations in the proportions of cells, fibres, and matrix as well as in the chemical nature of their mucopolysaccharides (Meyer & Rapport, 1951; Dische, Danilczenko, & Zelmenis, 1958) and in their behaviour *in vitro* (Parker, 1932).

Several considerations suggest that the fibroblasts exert their characteristic effects on epithelial differentiation by means of the different intercellular materials that they produce, and, more specifically, by means of the basement membrane. Firstly, intercellular material always intervenes between fibroblasts and epithelium, and since it is known to vary from tissue to tissue it may well provide the means whereby fibroblasts exercise their characteristic effects.

Wilde's (1960) demonstration that 'extracellular material' is able to influence differentiation in early amphibian embryos lends support to this idea. Secondly, the reaction of isolated epidermis to cell-free intercellular material (McLoughlin, 1961*c*), and its similar response to whole mesenchyme, suggest that the basement membrane is formed from mesenchymal intercellular material under the influence of the epidermis and that it affects the orientation of the epithelium. Thirdly, the basement membrane varies with the underlying connective tissue, for it is not visible beneath epidermis on cartilage or heart myoblasts and must therefore be either altered or absent. On myoblasts, the epidermis is both oriented and capable of survival, which implies that a membrane is present though too thin to be seen. That cartilage, on the other hand, fails to supply a satisfactory basement membrane, is suggested by the disorientation and degeneration of epidermis implanted on it, and also by some results of Trinkaus (1961). He found that disaggregated cells from the epithelial pigment layer of the chick retina, when mixed with perichondrium, remain as isolated disoriented units, whereas in combination with kidney they form typical epithelial tubules. It is important to remark that the term 'basement membrane' is often used to describe different structures in electron and light microscopy. In this paper it is used in the light microscopist's sense of a lamella, lying between epithelium and mesenchyme, that stains red with the PAS method and precipitates silver, i.e. that contains mucopolysaccharide and fine collagen fibrils (reticulin). It always stains for neutral mucopolysaccharides whatever the staining reaction of the general matrix. Ozzello & Speer (1958) found acid mucopolysaccharides in the basement membrane of the human mammary gland only at sites of carcinomatous invasion.

Finally, the different effects of gastric, limb, and heart fibroblasts on the spreading of implanted epidermis suggest that the basement membranes they provide differ, in spite of their similar appearance in the light microscope. Proventriculus and gizzard promote epithelial spreading, limb permits it, and heart fibroblasts strongly inhibit it; thus the ability of the basal cells to glide over their basement membrane varies according to the type of mesenchyme with which they are associated. Experiments in which gizzard epithelium was implanted on limb mesenchyme (p. 397) show that this is a result of differences in the basement membrane rather than in the cells themselves.

There is a correlation between the degree to which each mesenchyme inhibits spreading and the ability of the epidermis to keratinize on that mesenchyme, e.g. heart fibroblasts strongly inhibit spreading and promote keratinization, whereas gizzard promotes spreading and inhibits keratinization. That this connexion between the spreading and differentiation of the epithelium must be something more subtle than a purely mechanical effect is shown by two observations. Firstly, epidermis on gizzard mesenchyme may become as thickened as in keratinizing control cultures, and yet continues to secrete mucus (cf. Plate 2, fig. 13); and secondly, the cells even of the epidermal basal layer

differ when placed on the various mesenchymes, e.g. on gizzard the basal cells contain few tonofibrils, on limb tonofibrils are plentiful, and on heart fibroblasts the basal cells are packed with them.

The possibility that epithelio-mesenchymal interactions continue into adult life, and their relation to pathology, have been discussed briefly elsewhere (McLoughlin, 1960). Recently, Lasfargues, Murray, & Moore (1960) have found that the mouse mammary carcinoma agent will not multiply in cultures of pure mammary gland epithelium, but grows in explants of mammary epithelium and stroma together. As this observation was made on adult tissue, it indicates that epithelio-mesenchymal interactions persist in the adult. This conclusion is further supported by the observation of Dawe (1960) that in mice the polyoma virus causes carcinomata of organs, such as salivary gland and thymus, where epithelio-mesenchymal interactions during development are either known or suspected.

SUMMARY

1. Experiments were undertaken to investigate the degree to which the underlying mesenchyme influences the differentiation of an epithelium. Isolated epidermis of the 5-day chick embryo was explanted *in vitro* in contact with three different types of mesenchyme from chick embryos of the same age. These mesenchymes were derived from the gizzard, proventriculus, and heart. In a control series epidermis was re-implanted on limb mesenchyme. In each combination the epidermis behaved in a different and characteristic way.

2. The cellular processes by which an isolated epithelium becomes established on mesenchyme *in vitro* are described.

3. In the control series, epidermis re-implanted on limb mesenchyme keratinized normally, but in contact with cartilage it degenerated.

4. On gizzard mesenchyme epidermis is prevented from keratinizing and is induced to secrete mucus and sometimes to become ciliated.

5. On proventriculus mesenchyme epidermis is initially prevented from keratinizing and secretes mucus, but after about 7 days it reverts to its normal differentiation and keratinizes.

6. Explants of heart mesenchyme become subdivided into two regions: a central myoblastic area surrounded by a zone of fibroblastic outgrowth. On the central region of myoblasts the epidermis is prevented from keratinizing and spreads to a single-layered squamous epithelium. On heart fibroblasts, however, the epidermis keratinizes more densely than in the controls.

7. The four types of mesenchyme differ in the nature of the intercellular material that they contain, and in the amount of epithelial spreading they permit.

8. The various effects of the mesenchyme are probably exerted specifically by the fibroblasts, which arrange themselves in contact with the epithelium in most types of culture.

9. These epithelio-mesenchymal interactions are discussed in relation to the

initiation and maintenance of embryonic and adult differentiation. It is suggested that the intercellular material of the mesenchyme may participate in the mechanisms underlying these reactions.

RÉSUMÉ

L'importance des facteurs du mésenchyme dans la différenciation de l'épiderme de Poulet

II. *Modification de la différenciation épidermique par contact avec différents types de mésenchymes*

1. Des expériences ont été entreprises pour savoir jusqu'à quel degré le mésenchyme sous-jacent influence la différenciation d'un épithélium. De l'épiderme d'embryon de Poulet de 5 jours a été associé, en culture *in vitro*, à 3 types différents de mésenchymes embryonnaires de Poulet de même âge. Ces mésenchymes proviennent du gésier, du proventricule et du cœur. Dans des séries témoins, l'épiderme a été réimplanté sur du mésenchyme de membre. L'épiderme se comporte d'une manière différente et caractéristique selon la combinaison réalisée.

2. On décrit les processus cellulaires suivant lesquels l'épithélium isolé s'établit sur le mésenchyme, *in vitro*.

3. Dans les séries témoins l'épiderme se kératinise normalement s'il est réimplanté sur du mésenchyme de membre, mais il dégénère au contact de cartilage.

4. Le mésenchyme de gésier empêche l'épiderme associé de se kératiniser et l'induit à sécréter du mucus, et quelquefois à devenir cilié.

5. Le mésenchyme de proventricule empêche d'abord l'épiderme associé de se kératiniser et le fait sécréter du mucus, mais, après 7 jours, cet épiderme retourne à sa différenciation normale et se kératinise.

6. Des explants de mésenchyme cardiaque différencient deux zones: une aire centrale myoblastique qu'entoure une zone de croissance fibroblastique. La région centrale des myoblastes empêche l'épiderme de se kératiniser; celui-ci s'étale en un épithélium squameux formé d'une seule assise de cellules. Sur les fibroblastes de cœur, par contre, l'épiderme se kératinise d'une manière plus dense que chez les témoins.

7. Les 4 types de mésenchyme diffèrent par la nature de leur matériel intercellulaire, et par la propriété qu'ils ont de permettre l'étalement épithélial en quantités différentes.

8. Les fibroblastes qui s'organisent au contact de l'épithélium dans la plupart des types de culture, sont probablement responsables des différents effets du mésenchyme.

9. La discussion porte sur les interactions épithélium-mésenchyme relatives au départ et au maintien de la différenciation embryonnaire et adulte. On

suggère que le matériel intercellulaire du mésenchyme puisse participer aux mécanismes qui sont à la base de ces réactions.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Living culture of epidermis on limb mesenchyme, 7 days *in vitro*. The epidermis has formed a keratinizing cyst (*C*). Cartilage black. Swathes of myoblasts are visible. ×24.

Fig. 2. Epidermis on limb mesenchyme after 24 hours *in vitro*. The epidermal nodule is surrounded by phagocytic peridermal cells (*P*). Basal cells (*B*) become oriented in contact with the mesenchyme. See also fig. 4. Meyer's acid haemalum and Alcian blue. ×240.

Fig. 3. Epidermal cells situated above the basal layer send processes (*P*) down to the basement membrane. On the left, 2 cells send down entwined processes (*PP*). Iron haematoylin. ×1440.

Fig. 4. High-power view of part of the same section as in fig. 2. The processes of more distal cells sometimes contain a spiral filament (*T*). Meyer's acid haemalum and Alcian blue. ×860.

Fig. 5. Processes of distal cells terminate in small feet (*F*) on the basement membrane (*BM*) which is cut somewhat obliquely. Iron haematoxylin. ×1440.

Fig. 6. The normal 2-layered structure of the epidermis is reconstructed after 48 hours *in vitro*. Iron haematoxylin. ×850.

Plate 2

Fig. 7. Typical keratinization in a cyst of epidermis on limb mesenchyme after 7 days *in vitro*. Note that the fibroblasts have been selectively attracted to the epidermis. Trevan's method. ×210.

Fig. 8. Fluid-filled cyst of epidermis on limb mesenchyme after 12 days *in vitro*. Keratinization in such cysts is delayed but normal. On the left a small region of the epidermis is in contact with perichondrium, which causes a localized degeneration. Azan. ×250.

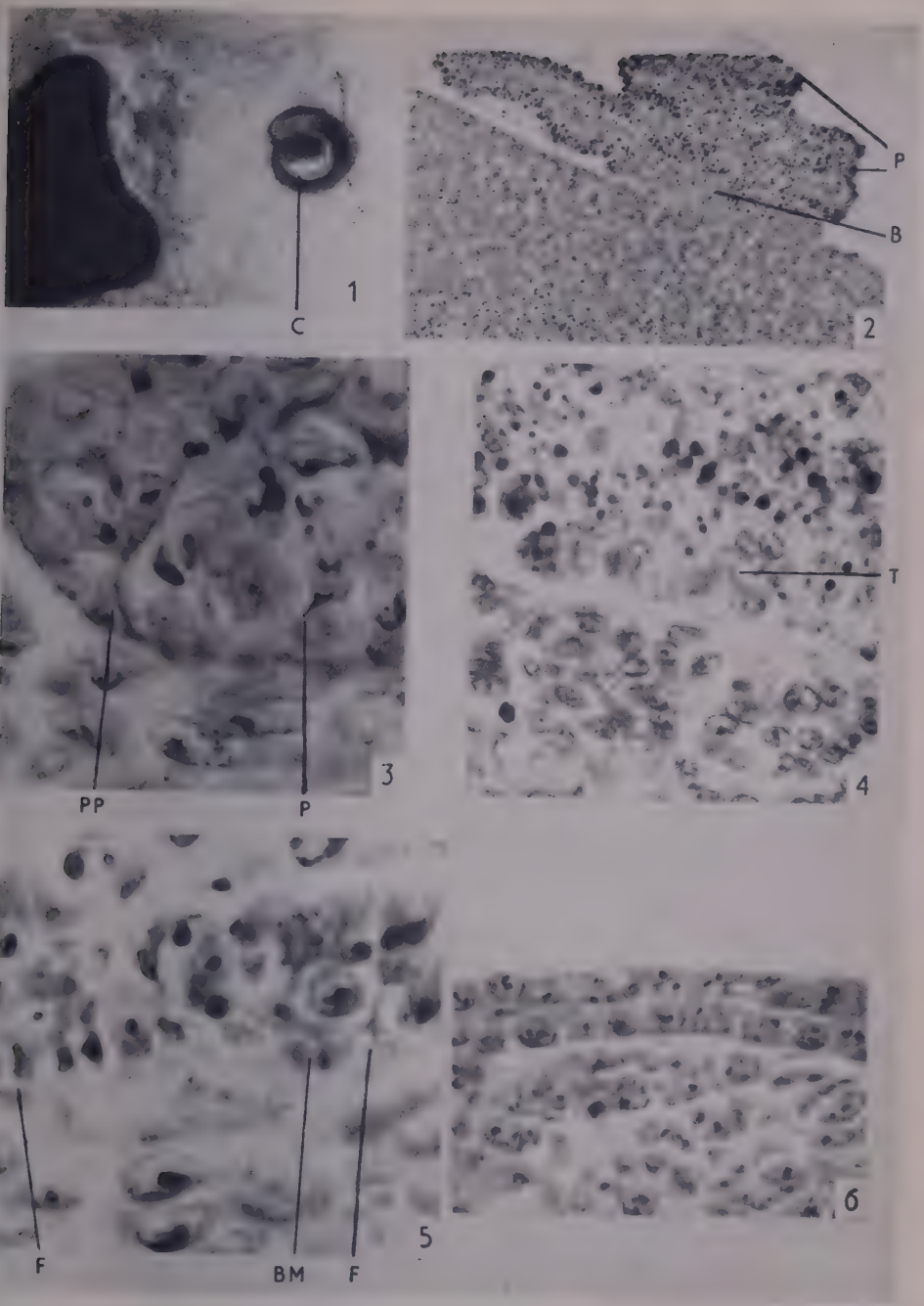
Fig. 9. Living culture of epidermis on gizzard mesenchyme after 5 days *in vitro*. The refractile lining of the epithelial cyst (*C*) is due to mucus secretion. Cords of epidermis extend into the medium. Note the large autonomic ganglion (*G*) with radiating bundles of nerve fibres. The dark object obscuring the lower right-hand part of the cyst is an artefact. ×24.

Fig. 10. Epidermis on gizzard mesenchyme after 5 days *in vitro* forms a 2-layered columnar epithelium. A little mucus (*M*) is visible as a dark lining in the upper part of the lumen. Iron haematoxylin. ×228.

Fig. 11. Part of a distended cyst of epidermis on gizzard mesenchyme after 12 days *in vitro*. Mucus formed by the innermost epidermal layer stains darkly blue-green with Alcian blue. ×750.

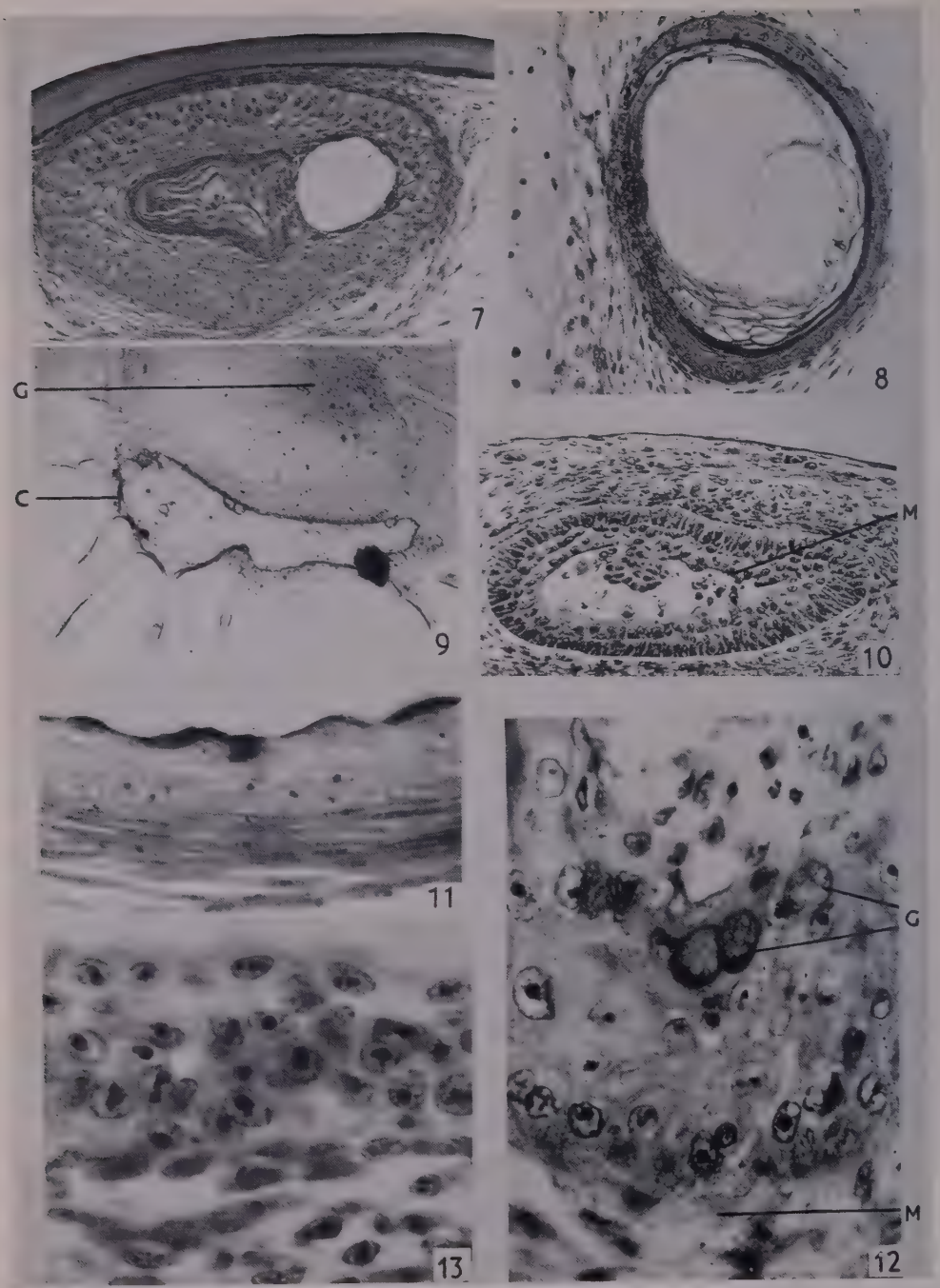
Fig. 12. Part of a collapsed cyst of epidermis on gizzard mesenchyme after 11 days *in vitro*. Degenerating peridermal cells appear in the upper part of the picture; below them the thickened epidermis contains 4 goblet cells (*G*) of basal origin. Mesenchyme (*M*) in the lowest part of the picture. Meyer's acid haemalum and Alcian blue. ×750.

Fig. 13. Epidermis arranged as a sheet on gizzard mesenchyme has become ciliated. Note that the epidermis is 4-cell-layers thick, but shows no sign of keratinizing at 6 days *in vitro*. Azan. ×1000.



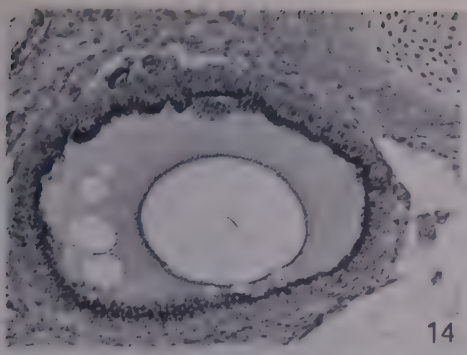
C. B. McLOUGHLIN

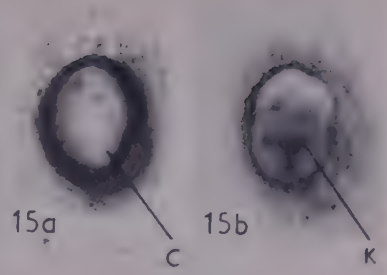
Plate 1

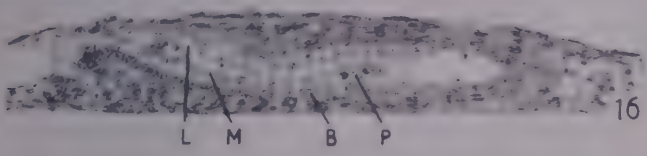


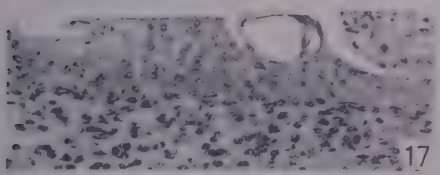
C. B. McLOUGHLIN

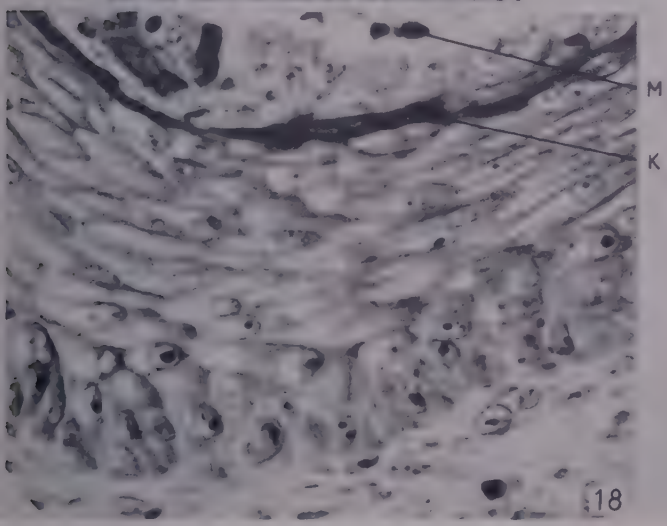
Plate 2





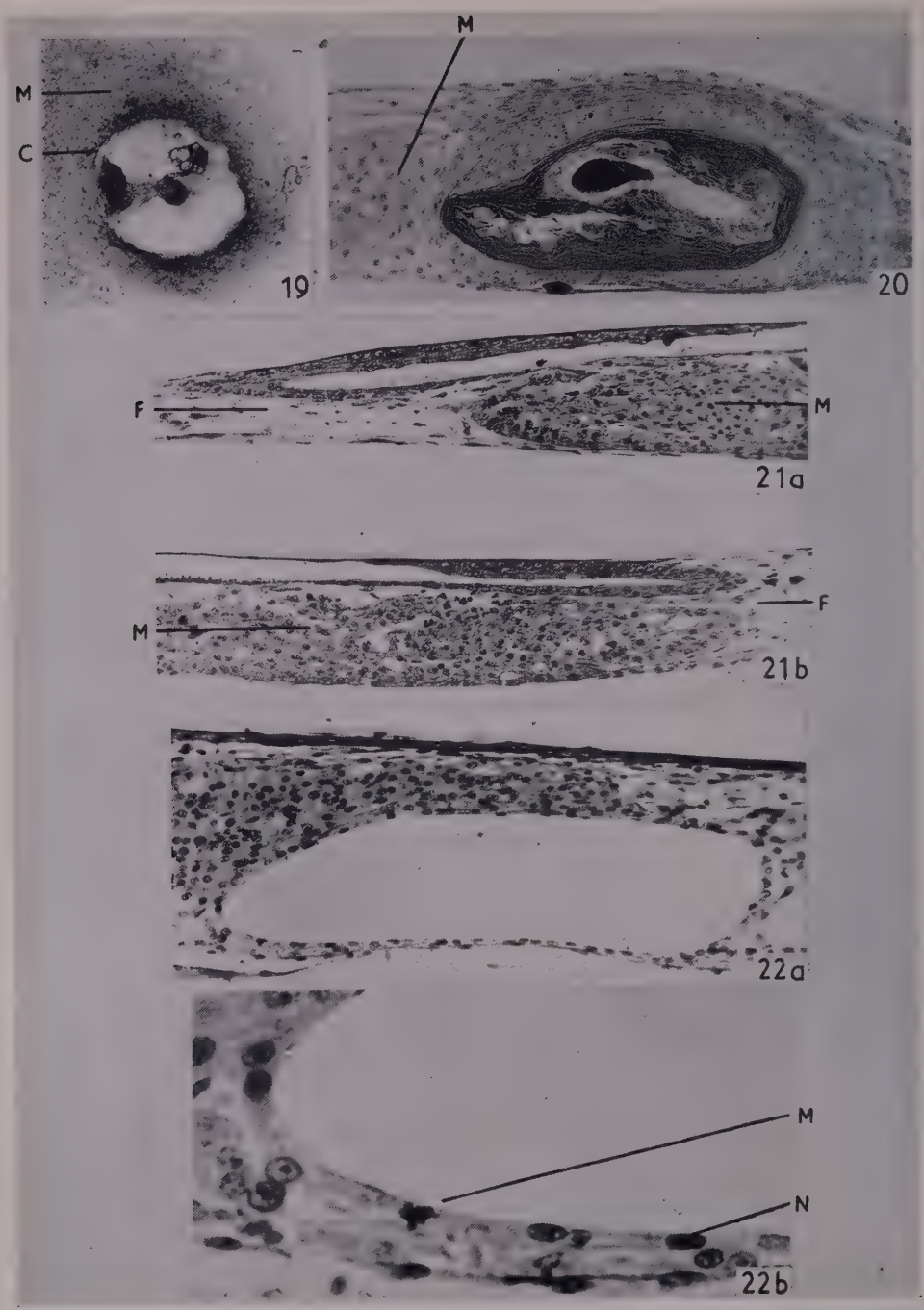






C. B. McLOUGHLIN

Plate 3



C. B. McLOUGHLIN

Plate 4

Plate 3

Fig. 14. Gizzard epithelium on limb mesenchyme after 7 days *in vitro* continues to secrete mucus, i.e. its differentiation is unaltered, but the limb mesenchyme causes it to roll up into a cyst instead of spreading in a sheet, as gastric epithelium usually does. Note the nodule of cartilage in the upper right-hand corner. Meyer's acid haemalum and mucicarmine. ×140.

Fig. 15. Living cultures of epidermis on proventriculus mesenchyme. *a*, after 5 days *in vitro* the epidermis has formed a cyst (*c*) which secretes mucus and fluid. *b*, after 8 days *in vitro* the epidermis thickens to begin forming keratin (*K*). ×24.

Fig. 16. Epidermis on proventriculus mesenchyme after 2 days *in vitro* has reconstructed into a 2-layered epithelium arranged as a cyst around a flattened lumen (*L*). The inner peridermal cells carry the marker carbon particles (*P*), and are seated on a single layer of darker basal cells (*B*). A little mucus (*M*) is already visible on the surface of the peridermal cells. Meyer's acid haemalum and mucicarmine. ×270.

Fig. 17. Epidermis on proventriculus mesenchyme after 7 days *in vitro* thickens prior to keratinizing. Meyer's acid haemalum and Alcian blue. ×270.

Fig. 18. Part of a culture of epidermis on proventriculus mesenchyme, 10 days *in vitro*. The epidermis is keratinizing normally, and the previously secreted mucus (*M*) is carried into the centre of the cyst. Iron haematoxylin. ×750.

Plate 4

Fig. 19. Living culture of epidermis on heart myoblasts (*M*) after 6 days *in vitro*. The epithelium forms a thin-walled, fluid-filled cyst (*C*). An epidermal strand stretching across the cyst, out of contact with the myoblasts, is keratinizing (three dark areas); this interpretation was confirmed histologically. ×24.

Fig. 20. An epidermal cyst on heart fibroblasts after 8 days *in vitro* forms compact keratin. The boundary of the myoblastic region (*M*) appears on the left. Azan. ×180.

Fig. 21. Section through the two ends of a sheet of epidermis on heart mesenchyme, 2 days *in vitro*. The epidermis has spread thinly over the entire myoblastic region (*M*), but at the boundary with the fibroblastic region (*F*) it is reflected and piles up to form a stratified squamous epithelium. Iron haematoxylin. ×230.

Fig. 22*a*. Epidermis on heart myoblasts, 8 days *in vitro*, remains as a single squamous layer. Azan staining shows the absence of fibroblasts in the lower wall. ×180.

Fig. 22*b*. High-power view of the lower left part of fig. 22*a*, showing the single layer of flattened epidermal cells, whose nuclei (*N*) appear darker than those of the surrounding myoblasts. A mitosis (*M*) indicates that the epidermis is still healthy. Azan. ×820.

Plate 1, fig. 3; Plate 2, figs. 7 and 11; and Plate 4, figs. 20 and 22*a* are reprinted from 'Biological Approaches to Cancer Chemotherapy' by kind permission of the publishers, Academic Press Inc. (London) Ltd.

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Réaction de l'embryon de Poulet au bleu trypan

par F. STÉPHAN[1] *et* B. SUTTER[2]

Laboratoire de Zoologie et d'Embryologie expérimentale, Faculté des Sciences, Strasbourg

AVEC DEUX PLANCHES

En 1941, Ancel & Lallemand signalaient que le bleu trypan était tératogène pour l'embryon de Poulet. Depuis lors de nombreux chercheurs ont utilisé le même produit avec des résultats quelque peu différents suivant les groupes de vertébrés testés: Oiseaux, Mammifères et Batraciens.

Ancel & Lallemand avaient disposé le colorant sur le blastoderme de 48 heures d'incubation. Ils obtinrent au bout de 24 heures des hémorragies siégeant de préférence dans la région postérieure de l'axe embryonnaire. Diverses autres substances leur donnèrent des résultats similaires, notamment le sulfate de strychnine choisi par Stoll (1941) pour étudier la genèse et la structure de ces hématomes. Ancel (1950) rend ces lésions primaires responsables des anomalies, jugées secondaires, qui se manifestent à leur niveau chez des embryons plus âgés: cœlosomie pour les hémorragies de la paroi latéro-ventrale, réductions variables de l'extrémité caudale (anourie, brachyourie) ou des membres postérieurs. Il n'exclut pas cependant la possibilité d'un mécanisme direct dans la production des monstruosités plus tardives, car certains anoures provenant des mêmes séries expérimentales n'avaient pas été porteurs d'hémorragies. Des anomalies diverses telles que cœlosomie, microphthalmie, anophthalmie, déficiences du bec ou des membres postérieurs, et surtout anourie, se présentent chez les embryons traités à 24 et 48 heures par Beaudoin & Wilson (1958). Enfin tout récemment, Mulherkar (1960) décrit chez des embryons de 2 jours environ, traités à des stades allant de la ligne primitive à la plaque médullaire, des irrégularités somitiques et des soufflettes au niveau desquelles le mésoderme axial est réduit, la chorde notamment.

Les anomalies induites par le bleu trypan chez les embryons de Mammifères (il y a une floraison de travaux concernant les Rongeurs) sont pour une part celles précédemment trouvées chez l'embryon de Poulet âgé, mais il en est aussi d'autres qui touchent le cœur et les gros vaisseaux, ainsi que le système nerveux central, le cerveau en particulier qui est souvent hypertrophié (hydrocéphalie, exencéphalie, etc.).

Les embryons de Batraciens (Waddington & Perry, 1956) peuvent présenter

[1] *Author's address*: Institut de Zoologie, 12, rue de l'Université, Strasbourg, France.

[2] *Author's address*: Institut de Physiologie, 2, rue Boussingault, Strasbourg, France.

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des blocages de la gastrulation à des degrés divers, des exogastrulations plus ou moins complètes, des dégénérescences tissulaires (sauf pour l'endoderme) et des œdèmes, des inhibitions du bourgeon caudal, des réductions et suppressions de la chorde au profit des somites et, chose qui contraste avec les observations faites chez les Mammifères, de la microcéphalie. Celle-ci est attribuée à un blocage tardif de la gastrulation.

Ainsi donc, en l'absence de données plus complètes concernant l'embryon de Poulet, ce dernier semblait se comporter d'une manière spéciale sur deux points. Ici pas de malformation caractérisée du système nerveux, pas d'anomalie cardio-vasculaire. Ceci nous avait incités à entreprendre de nouvelles recherches dont les premiers résultats seront rapportés dans la présente note. Outre les faits déjà signalés par Ancel & Lallemand, nous avons relevé des dispositions vasculaires anormales, des strophosomies et, comme Mulherkar, des irrégularités dans la segmentation.

MÉTHODES

Toutes les expériences ont été faites sur des embryons de race Leghorn blanche, à diverses périodes de l'année 1959 et au début de 1960. Les œufs furent incubés à la température de 37,5° C. Ils furent ouverts suivant la technique d'Ét. Wolff. La fenêtre pratiquée dans la coquille était refermée par un bout de ruban adhésif. L'évolution des embryons était suivie de jour en jour.

Le bleu trypan, qui provient des Établissements Hollborn u. Söhne de Leipzig, fut dissous au 1/1000 dans l'eau distillée ou le liquide physiologique de Tyrode. L'eau distillée servit au début de ces recherches, le liquide physiologique eut la préférence par la suite, car il permet une meilleure survie des embryons. La dose administrée variait entre 0,01 à 0,05 mg. de colorant (pour 0.01 à 0,05 cm^3 de solution). Les embryons devant servir de contrôles recevaient en même quantité de l'eau distillée ou du liquide physiologique suivant le cas. D'autres étaient simplement ouverts et refermés.

Dans les premières expériences, le colorant était ou déposé sur l'embryon ou injecté dans le vitellus, auquel cas il remonte en quelques secondes et s'accumule sous le blastoderme. Dans une deuxième série, il fut introduit directement dans la cavité sous-germinale. Par cette méthode on peut, en opérant avec douceur, localiser grossièrement le produit sous une région donnée de l'embryon, sous sa moitié postérieure par exemple. Enfin, dans une 3ème série d'essais, le colorant imprégnait un petit bloc de gélose placé sur l'embryon en un point bien localisé. La dose était dans ce cas souvent moindre que celle indiquée précédemment. Deux cent quarante-huit embryons ont été traités au second jour de l'incubation suivant l'une ou l'autre de ces modalités; 49 autres ont reçu, entre 48 et 72 heures d'incubation, un cube de gélose-bleu trypan sur la région branchiale. Les embryons ont été fixés au formol picroacétique et dessinés à la chambre claire. Ceux destinés à l'étude histologique ont été coupés à 5 μ et colorés à l'hématoxyline-éosine.

RÉSULTATS

Les malformations apparues à la suite du traitement au bleu trypan sont les unes précoces — il s'agit d'œdèmes et d'irrégularités somitiques — les autres tardives: réduction de l'extrémité caudale et des membres postérieurs, flexions anormales de la colonne vertébrale troncale et cœlosomie, anomalies vasculaires. Elles seront décrites dans cet ordre. Des anomalies du bec, des microphthalmies ont été notées sporadiquement; elles ne seront pas considérées.

Œdèmes

Les œdèmes ont été signalés déjà par Ancel & Lallemand, sur des embryons de 3 jours traités la veille, et par Mulherkar sur des embryons bien plus jeunes traités au stade plaque neurale, voire plus tôt. Nos interventions ont été faites au 2ème jour, alors que les embryons avaient formé 4–20 somites, par l'une ou l'autre des méthodes indiquées, sans qu'il y ait pour cela des différences essentielles dans les résultats.

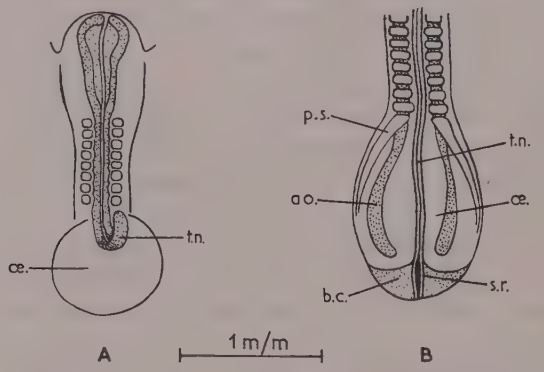


FIG. 1. Œdèmes axiaux. *ao.*, aorte dorsale; *b.c.*, bourgeon caudal; *œ.*, œdème; *p.s.*, plaque somitique; *s.r.*, sinus rhomboïdal; *t.n.*, tube neural.

A ce stade, les embryons sont fragiles, la seule ouverture des œufs est une cause de mortalité. La plupart succombent dans les 24 heures qui suivent le traitement; parmi eux beaucoup sont porteurs d'une vésicule en arrière de la région segmentée. Tel embryon (fig. 1A) qui a reçu une goutte de bleu trypan dans l'eau distillée (0,05 cm^3 environ) au stade de 5 paires de somites, est mort au bout de quelques heures après avoir formé 3 autres paires de somites — et une vésicule. Tel autre qui a reçu la même dose dans la cavité sous-germinale à 11 somites, vivait encore 24 heures après. Il avait alors 26 paires de somites et à leur suite la vésicule, hémorragique cette fois, les aortes postérieures s'étant rompues. Un autre enfin a reçu un cube de gélose coloré sur sa région postérieure. Après quelques heures celle-ci était gonflée par l'œdème entre le 27ème et dernier

somite et le tissu dense du bourgeon caudal; les plaques somitiques étaient rejetées contre les parois latérales et les aortes apparaissaient par transparence plus près de l'axe embryonnaire (fig. 1B). Ajoutons que des œdèmes beaucoup moins prononcés siègent parfois dans la paroi latéro-ventrale (cf. Ancel, 1950).

L'examen histologique montre que la poche se développe entre ectoderme et entoderme distendus (Planche 1). La région des derniers somites est parfois légèrement touchée, les somites y apparaissent creux. Dans la poche, le tissu nerveux garde sa forme, s'aplatissant seulement vers son extrémité en une lame pleine. La chorde s'interrompt plus tôt que le tube nerveux dont elle est légèrement écartée. Les plaques somitiques s'amenuisent vers l'arrière et s'écartent l'une de l'autre. Aux endroits où la chorde fait défaut on ne trouve plus que des lames mésodermiques délimitant l'œdème axial d'avec le cœlome. La limite postérieure de la poche est imposée par le tissu dense du bourgeon caudal. Chez les embryons qui n'ont pas encore développé ce bourgeon, elle peut déborder sur le territoire extra-embryonnaire.

Les œdèmes, hémorragiques ou non, apparaissent dans toutes les séries ayant reçu du bleu trypan et seulement dans celles-là (tableau 1). Sur les 248 embryons traités au second jour, 83, soit 33 pour cent, sont porteurs d'œdèmes. On relève 23, soit 9 pour cent, d'œdèmes simples et 60, soit 24 pour cent, d'œdèmes hémorragiques. La fréquence de la malformation est plus élevée lorsque le colorant est administré dans l'eau distillée (54 pour cent) que lorsqu'il l'est en liquide physiologique (29 et 21 pour cent), tandis que le nombre d'embryons morts précocement sans lésion apparente est le même à peu près dans l'un et l'autre cas.

Dans les expériences d'Ancel & Lallemand, faites sur des embryons de 48 heures pourvus d'une circulation active, la première manifestation du colorant était une hémorragie partant des aortes postérieures et se développant en une vésicule du type décrit ici. A ces auteurs l'hémorragie apparaissait comme primaire. Les résultats de Mulherkar (1960) et les nôtres indiquent qu'à des stades plus jeunes il se constitue un œdème, qui peut se remplir de sang ultérieurement. L'hémorragie est certainement secondaire dans ce cas; peut-être l'est-elle aussi à 3 jours, mais alors elle apparaît pratiquement avec l'œdème et, plus spectaculaire que lui, elle le masque.

Lésions des somites

Les irrégularités somitiques ont été observées bien moins souvent que les œdèmes; nous n'en avons trouvé que 18 cas nets pour les 248 embryons traités. Ils se rencontrent parmi les embryons ayant de 15 à 26 somites au moment de l'intervention.

Alors que les somites normaux se présentent en vue dorsale sous l'aspect de briques empilées et qu'ils se moulent sur leurs voisins et sur le système nerveux, les somites lésés n'ont plus en général de tels rapports et toujours leurs angles sont arrondis. Tantôt ces somites sont raccourcis, comme tassés (fig. 2D) et

TABLEAU 1

Résultats comparatifs de la production d'œdèmes par le bleu trypan (BT) administré en eau distillée (BT/E. dist.), en liquide de Tyrode (BT/Tyrode) ou en un bloc de gélose dans le liquide physiologique (BT/gél. Tyr.)

Méthode	*E. dist.*		*Tyrode*		*B.T. E. dist.*		*B.T. Tyrode*		*B.T. gél. Tyr.*		*Total B.T.*	
	Nombre d'embryons	%	*Nombre d'embryons*	%	*Nombre d'embryons*	%	*Nombre d'embryons*	%	*Nombre d'embryons*		*Nombre d'embryons*	%
Morts dans les 24 heures:												
Avec œdème	0	0	0	0	35	50	23	24	17	21	75	30
Sans œdème	4	15	1	5	18	26	21	22	18	22	57	23
TOTAL	4	15	1	5	53	76	44	46	35	43	132	53
Vivants 24 heures après traitement:												
Avec œdème	0	0	0	0	3	4	5	5	0	0	8	3
Sans œdème	22	85	19	95	14	20	47	49	47	57	108	44
TOTAL	22	85	19	95	17	24	52	54	47	57	116	47
NOMBRE TOTAL D'EMBRYONS	26		20		70		96		82		248	

tantôt ils se réduisent à des sphérules de taille variable (fig. 2 A, B). D'autres fois au contraire ils sont anormalement longs (fig. 2 C, E) et donnent l'impression d'être formés de plusieurs éléments coalescents. Souvent la correspondance droite–gauche cesse d'être respectée en ce qui concerne la disposition des somites ou le moment de leur individualisation.

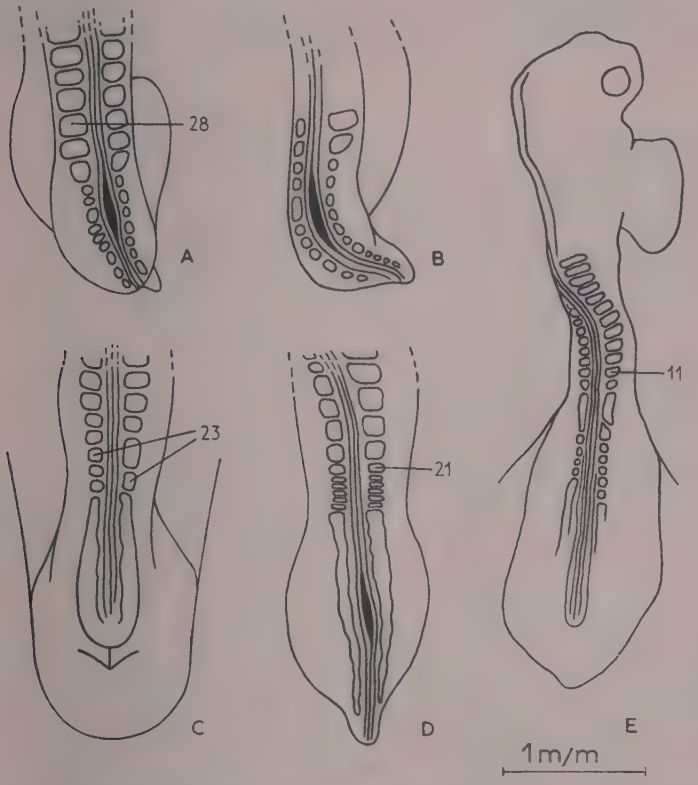


FIG. 2. Somites anormaux. Les nombres indiquent les numéros d'ordre des somites.

Notons qu'un tiers de ces embryons présente 24 heures après le traitement un retard dans la segmentation de 8–15 paires de somites, ce qui représente 8–15 heures, la segmentation progressant dans la période considérée à l'allure moyenne de 1 paire de somites par heure. Des retards analogues ont été observés aussi sur d'autres embryons traités qui ne sont pas atteints de malformations somitiques. Il reste à souligner que les somites anormaux n'apparaissent pas immédiatement, mais qu'il se forme encore 3–6 paires de somites normaux après le dépôt du colorant. Nous reviendrons sur ce point dans la discussion.

La réduction des somites est bien visible sur les préparations histologiques

(Planche 2). Ils sont entourés d'un grand espace vide dans lequel ils semblent parfois flotter. Dans une section transversale un somite normal couvre à peu près la surface du tube nerveux, un somite réduit peut n'en couvrir que la moitié. Le système nerveux, la chorde, les plaques latérales et l'endoderme ne paraissent pas affectés, mais l'ectoderme est anormalement pâle, lacunaire. L'arrangement cellulaire typique est conservé, même dans les somites; les cellules semblent saines. On a l'impression que leur nombre a diminué dans les somites. Cette impression a été vérifiée par un comptage effectué sur 20 coupes transverses

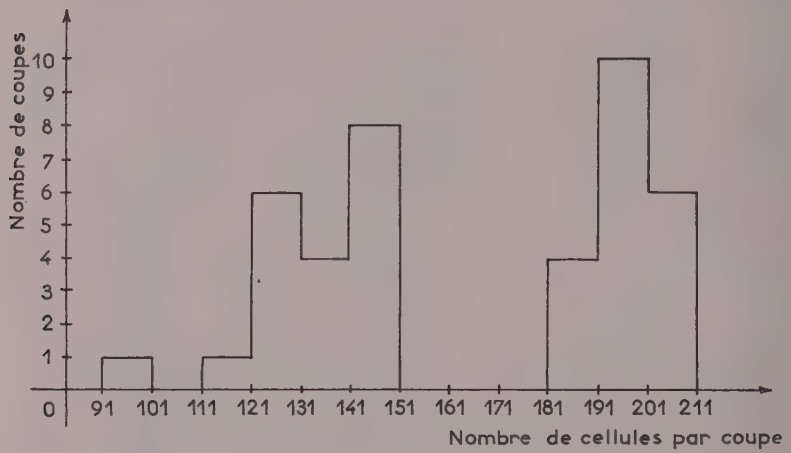


FIG. 3. Population cellulaire des somites normaux (à droite) et des somites réduits (à gauche) dans des coupes transverses de 5 μ.

passant au milieu d'autant de somites pris sur des embryons normaux et 20 coupes de somites réduits pris sur 2 embryons traités, celui de la figure 2C et un autre qui lui est semblable. Alors que les somites normaux ont de 180 à 210 cellules par coupe avec une moyenne de 196, les somites anormaux en ont de 90 à 150, en moyenne 143 (fig. 3). L'écart entre les deux groupes de valeurs doit tenir pour une part au choix des somites anormaux; on s'en est tenu à ceux dont la réduction était macroscopiquement visible. Il reste que les somites sont très diversement affectés par le colorant et que leur population cellulaire peut être réduite de moitié par rapport à la normale. Les plaques somitiques sont encore plus gravement atteintes que les somites. Leurs cellules sont dispersées comme dans un mésenchyme lâche, au point que les limites des plaques deviennent imprécises.

Malformations de la queue et des pattes

Des réductions de l'extrémité caudale ont été obtenues chez 74 pour cent des embryons (75 sur 102) traités au second jour de l'incubation par l'une ou l'autre des méthodes énumérées. Elles sont le plus souvent simples (62 cas, soit un peu

moins de 61 pour cent), allant de la brachyourie légère à l'anourie subtotale où la queue n'est plus représentée que par un cône minuscule dépourvu de squelette. Maiz chez 13 embryons, qui avaient tous reçu un cube de gélose-bleu trypan sur l'extrémité caudale, elles sont associées à des réductions des membres postérieurs. Ces derniers se rapprochent dans la mesure même où ils se réduisent. Un cas extrême est représenté par un symèle dont la patte unique ne se dédouble au bout que pour former deux petits cônes incurvés. Les malformations des membres postérieurs que nous avons observées sont ainsi liées à une réduction du squelette axial à leur niveau.

Malformations de la région troncale

Les diverses malformations groupées sous ce titre (et sous le suivant) ont été relevées dans un lot de 49 embryons qui ont reçu un bloc de gélose imprégné de colorant sur la région branchiale entre 48 et 72 heures d'incubation. Elles portent sur l'axe vertébral ou sur la paroi ventrale, le plus souvent sur les deux à la fois. Quinze embryons (30 pour cent) sont lordosiques, 15 autres sont strophosomes. Chez ceux-ci la moitié caudale du tronc est rabattue sur la face dorsale du thorax et les pattes appliquées de part et d'autre de la tête. L'anomalie avait été obtenue par Lallemand (1938) et par Gabriel (1946) à l'aide de la colchicine. Tous les strophosomes ainsi que 4 embryons lordosiques présentent en même temps une scoliose. La cœlosomie apparaît 27 fois (54 pour cent). Elle accompagne toujours la strophosomie et elle y est totale. Dans les 12 autres cas, dont 6 en association avec la lordose, il s'agit de cœlosomies supérieures et moyennes, avec hernie du cœur, de l'estomac et d'une partie de l'intestin.

Malformations des gros troncs artériels

Parmi les embryons traités au second jour, 23 seulement ont survécu jusqu'à 12 jours. Ils ont été sacrifiés à ce moment. Aucun n'a présenté de disposition artérielle atypique. D'autres embryons (49) reçurent alors de la gélose colorée sur la région branchiale au cours du 3e jour. Vingt-deux d'entre eux purent être examinés à 12 jours, dont neuf avaient des anomalies vasculaires.

Nous avons relevé les dispositions suivantes : persistance des 2 canaux carotidiens, de faible diamètre; persistance de ce canal à gauche avec l'arc 4 correspondant; persistance de ce même canal bien développé, une fois à droite, une autre fois à gauche avec suppression de la crosse normale (la crosse aortique emprunte le tronc brachiocéphalique et l'arc carotidien). Ces malformations avaient été produites antérieurement par ligature (Stéphan, 1952). Deux autres dispositions sont plus curieuses. Un embryon (fig. 4 A, B) possède à gauche une carotide issue de l'arc 4 et une vertébrale issue de l'arc 3 qui passe dorsalement à la carotide. Normalement l'artère vertébrale sort de la face dorso-latérale de la racine aortique au point où celle-ci reçoit l'arc 3 à sa face ventrale. Il est assez difficile de se représenter comment l'arc 3 a pu se détacher de la carotide tout en gardant le contact avec la vertébrale. L'autre embryon (fig. 4 C, D) a pareillement une carotide gauche venue de l'arc 4, la sous-clavière gauche est

présente mais non l'arc trois. A droite un canal carotidien sort de la crosse aortique, mais au lieu de rejoindre directement la carotide, il court parallèlement à elle. La carotide est ainsi dédoublée, la fusion des deux éléments se fait très progressivement au voisinage de la tête.

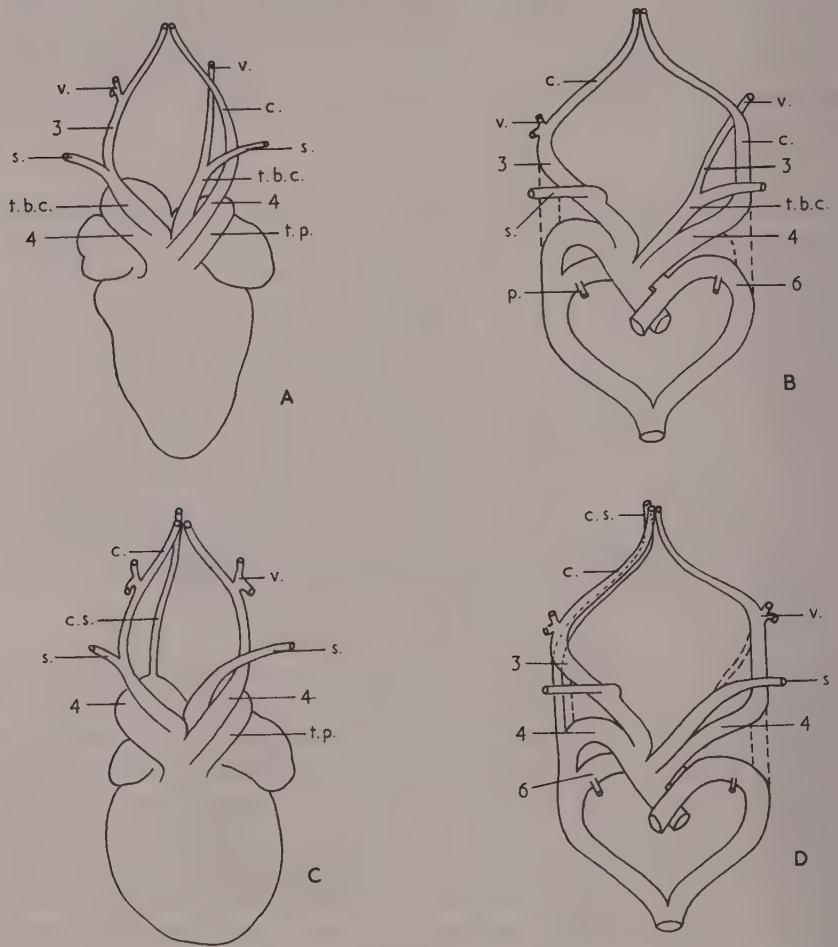


FIG. 4. Anomalies artérielles en vue ventrale. Les schémas B et D correspondent aux dessins A et C respectivement. *c.*, carotide; *c.s.*, carotide supplémentaire; *p.*, pulmonaire; *s.*, sous-clavière; *t.b.c.*, tronc brachiocéphalique; *t.p.*, tronc pulmonaire; *v.*, vertébrale; *3*, *4*, *6* arcs carotidiens systémique et pulmonaire.

Nous n'avons pas recherché les malformations cardiaques, mais leur existence est hautement probable, car après intervention directe sur les arcs aortiques, des déficiences septales se manifestent régulièrement dans la partie bulbaire de la cloison interventriculaire chaque fois que la crosse aortique normale vient à faire défaut (Stéphan, 1952).

DISCUSSION

L'un des résultats recherchés au départ de cette étude a été atteint, à savoir la production de malformations vasculaires. Ceci supprime une divergence qui semblait se manifester entre le comportement de l'embryon d'Oiseau d'une part, celui de Batracien et de Mammifère d'autre part. Il reste que le système nerveux du Poulet est peu sensible : très rares spina bifida, occasionnelles microphthalmies entraînant une déviation du bec supérieur.

Les artères voisines du cœur n'ont été touchées que dans des conditions très spéciales, dans le cas où le colorant était appliqué à leur voisinage au cours du 3ème jour de l'incubation. Le choix du moment joue un rôle important et peut-être intervient-il de manière assez précise. Chez les 2 embryons représentés, qui ont reçu le bleu trypan respectivement à 55 et 57 heures sous les arcs gauches, l'arc 3 gauche fut atteint. Il a disparu dans un cas, il prend un cours aberrant dans l'autre. Mais l'arc 4 a subsisté et il assure la relève de l'arc carotidien. Chez 2 autres embryons décrits l'arc systémique (4 droit) a régressé à la suite d'un traitement à droite à 65 heures. Les arcs carotidiens par contre se sont maintenus ; l'un d'eux fait même office de crosse aortique dans chaque cas. L'arc pulmonaire est toujours resté normal, mais il se développe bien après les autres, au 4ème jour, et aucune intervention n'a été tentée à ce moment. Il y a donc dans certains cas une relation nette entre d'une part le stade et le point d'intervention et d'autre part le type de malformation qui en résulte. Cette relation demande à être confirmée ; on peut penser qu'elle n'est pas purement fortuite, même si elle n'apparaît pas nettement dans d'autres expériences. Le cube de gélose se déplace quelquefois, d'autre part il exerce aussi une influence à distance. De toute façon l'effet doit dépendre de la diffusion plus ou moins grande du colorant.

Le mode d'action du colorant paraît se ramener ici à une inhibition de croissance. Mais les parois vasculaires sont encore purement endothéliales au moment du traitement. Peut-être se produit-il une compression du vaisseau, due à un œdème dans le mésenchyme voisin? Il est impossible d'en décider actuellement.

La cœlosomie dans ces expériences serait causée, selon Ancel (1950), par des hémorragies siégeant dans la future paroi ventrale. Mais la lordose et singulièrement sa forme aiguë, la strophosomie, interviennent probablement aussi, car lorsque l'axe de l'embryon plonge dans le vitellus, ses parois latérales se trouvent, par le fait même, rabattues vers le dos. En revanche ces mêmes parois en se refermant dans cette position pourraient amener les embryons lordosiques à la strophosomie. Nous serions donc tentés de croire que la strophosomie est primaire, due à la fragilité de l'axe embryonnaire, à un arrêt ou un ralentissement de la prolifération cellulaire au niveau du mésoderme axial. Le mécanisme ne serait pas sans analogie avec celui que déclenche la colchicine. Cette substance provoque la strophosomie en supprimant la croissance dans la région dorsale où elle est normalement la plus active à 3 jours (Ancel, 1950; Gabriel, 1946). La réduction

des régions postérieures de l'embryon est susceptible de la même interprétation.

Ce sont les réactions des embryons les plus jeunes qui étayent ces hypothèses. N'y voit-on pas l'appauvrissement du mésoderme axial en cellules, sans qu'il en résulte une 'mésodermisation' comme chez l'Amphibien (Waddington & Perry, 1956)? La chorde reste intacte, aussi épaisse que chez les témoins; les somites gardent leur bilatéralité, mais leur matériel cellulaire se réduit. Quel est le stade sensible? Si l'on se rappelle qu'après l'application de la substance il se forme encore 3–6 paires de somites normaux, ce qui prend 3–6 heures, alors que le colorant pénètre très vite, on est amené à conclure que les plaques somitiques ne sont pas touchées. Elles ont précisément la longueur de 3–6 somites, au moins jusqu'au stade à 12 paires de somites. Ce sont donc les centres somitiques eux-mêmes qui sont affectés, étant des centres de multiplication cellulaire intense (Spratt, 1957). Il en résulte des plaques somitiques lâches qui se fragmentent irrégulièrement en somites chétifs. Quant aux œdèmes, ils se développent dans la même région sensible. Ils signaleraient une atteinte plus brutale du mésoderme.

RÉSUMÉ

1. Des embryons de Poulet furent traités entre 24 et 72 heures d'incubation avec du bleu trypan en solution dans l'eau distillée ou le liquide physiologique, à des doses comprises entre 0,01 et 0,05 mg. de colorant.

2. Le traitement au deuxième jour produit (*a*) des œdèmes; (*b*) des anomalies somitiques consistant en asymétries, réductions de taille, appauvrissement en cellules; et (*c*) des agénésies de l'extrémité caudale.

3. Les réductions axiales peuvent s'étendre aux pattes et à la région sacrée, allant jusqu'à la symélie, lorsque le colorant, pris dans un bloc de gélose, a été déposé sur la partie postérieure insegmentée de jeunes embryons.

4. Les embryons plus âgés ont tous reçu de la gélose au bleu trypan sur leur région pharyngienne. Il en résulte (*a*) des cœlosomies, (*b*) des scolioses, lordoses, strophosomies, (*c*) des anomalies dans la distribution des grosses artères.

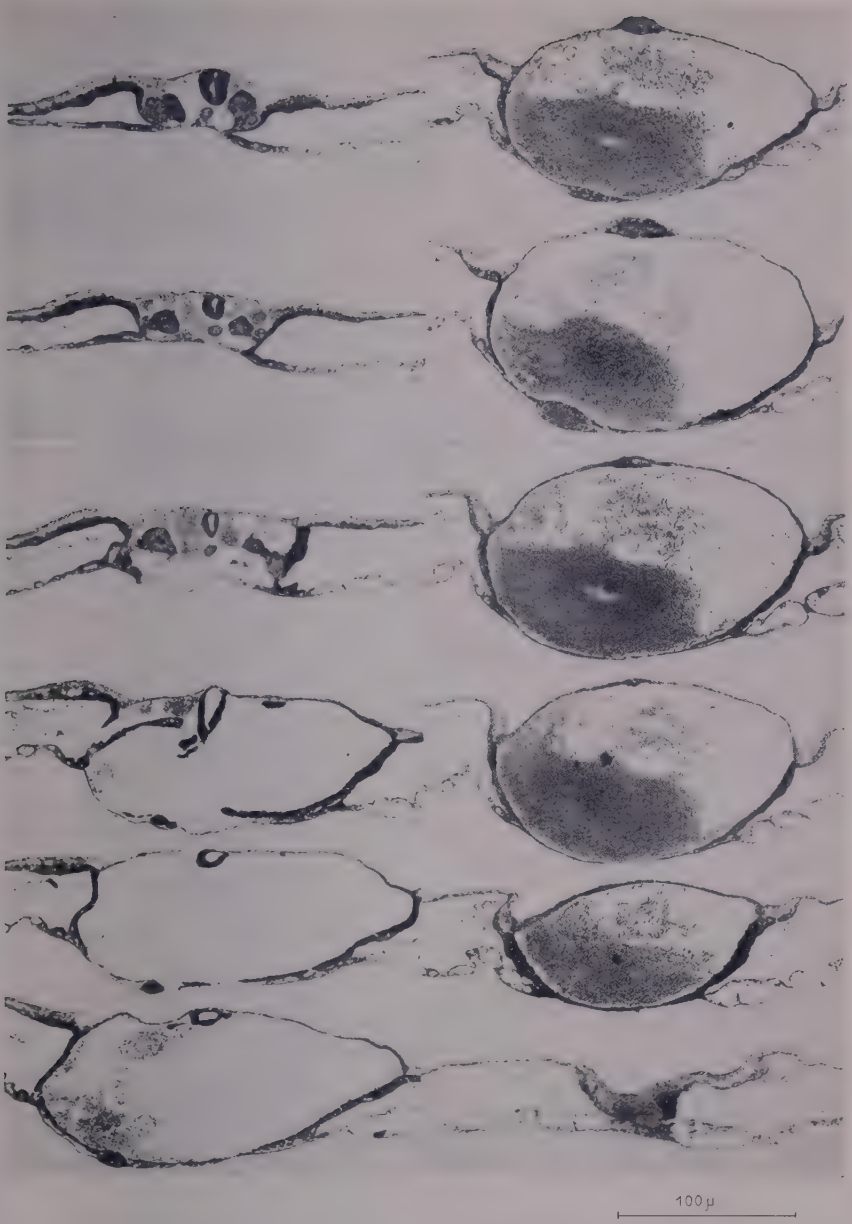
5. Il semble que le bleu trypan agisse aux stades jeunes en réduisant la prolifération cellulaire dans le mésoderme somitique. Son mode d'action aux stades avancés est discuté et quelques hypothèses sont proposées.

SUMMARY

1. Chick embryos were treated, between 24 and 72 hours of incubation, with doses of trypan blue, dissolved in distilled water or physiological saline, of between 0·01 and 0·05 mg. of the dye.

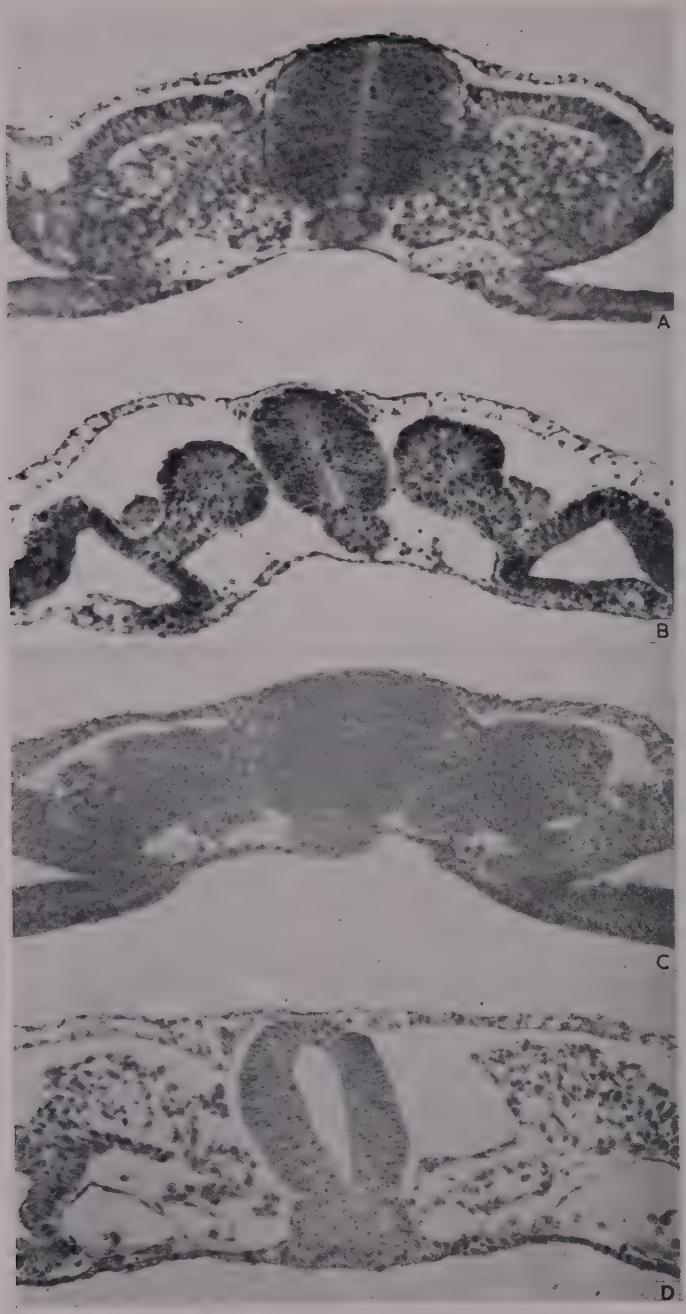
2. Treatment on the second day produced (*a*) oedema, (*b*) somite anomalies, consisting of asymmetry, reduction in size and number of cells, and (*c*) agenesis of the caudal extremity.

3. When the dye was included in a block of agar, and this was placed on the unsegmented posterior part of young embryos (second day), the axial reductions



F. STÉPHAN *et* B. SUTTER

Planche 1



F. STÉPHAN *et* B. SUTTER

Planche 2

could include the hind limbs and the sacral region, progressing as far as symmelia.

4. In all older embryos (third day) agar containing trypan blue was placed on the pharyngeal region. There resulted: (*a*) coelosomy, (*b*) scoliosis, lordosis and strophosomy, and (*c*) anomalies in the distribution of the great arteries.

5. Trypan blue apparently acts on young stages by reducing cellular proliferation in the somitic mesoderm. Its action in later stages is discussed and several hypotheses are proposed.

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EXPLICATION DES PLANCHES

Planche 1

Œdème hémorragique caudal. En lisant les coupes de haut en bas et en commençant par la colonne de gauche, on va de l'extrémité crâniale à l'extrémité caudale de l'œdème. Les coupes sont espacées à 50 μ.

Planche 2

Fig. A. Somites normaux.
Fig. B. Somites réduits.
Fig. C. Plaque somitique normale.
Fig. D. Plaque somitique réduite.

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Recherche sur la différenciation protéique au cours du développement des Amphibiens

par H. DENIS[1]

Laboratoire de Morphologie Animale, Université de Liège

INTRODUCTION

Au cours du développement embryonnaire, la différenciation protéique constitue probablement le phénomène fondamental qui entraîne la détermination des cellules et leur spécialisation en différents tissus. Diverses méthodes ont été utilisées en vue de suivre l'évolution des protéines au cours du développement.

La méthode immunologique a été appliquée pour la première fois à des œufs d'Amphibiens par Cooper (1946, 1948, 1950). Cet auteur constate la présence de 6 à 8 groupes antigéniques dans l'œuf indivis aussi bien que dans la jeune neurula de *Rana pipiens*. Par une méthode analogue, Flickinger & Nace (1952) observent l'apparition d'un nouvel antigène au stade bourgeon caudal. Spar (1953) montre l'existence d'antigènes propres à des stades déterminés (blastula, gastrula, neurula) et absents aux autres stades. Clayton (1953) met en évidence une synthèse de matériel antigénique d'abord entre le stade blastula et gastrula, ensuite avant la neurulation et enfin entre la neurulation et l'apparition du bourgeon caudal.

Plusieurs auteurs se sont également efforcés de séparer les protéines embryonnaires par la technique de l'électrophorèse. Par cette méthode, Flickinger & Nace (1952) observent une fraction protéique supplémentaire dans tous les extraits d'œufs et d'embryons plus âgés que le stade petit oocyte. Barth & Barth (1954) séparent la portion extractible du vitellus de *R. pipiens* en deux éléments, l'un phosphoré, l'autre non phosphoré. Selon Flickinger (1956), cette dernière fraction possède une activité phosphoprotéinephosphatasique et jouerait un rôle capital dans l'induction embryonnaire. Spiegel (1958, 1960) soumet à l'électrophorèse sur papier filtre des extraits d'œufs et d'embryons de *R. pipiens*. L'auteur obtient à partir de l'œuf vierge 7 bandes colorables par le bleu de bromophénol et une bande fluorescente qui ne semble pas de nature protéique. Au stade 21, trois de ces bandes ont disparu et la concentration totale en protéine soluble a diminué fortement.

Les résultats sérologiques obtenus jusqu'à présent donnent à penser que le développement des Amphibiens s'accompagne de l'apparition, à des moments variés, de nouvelles protéines. Si ces résultats n'ont pas été jusqu'ici confirmés

[1] *Author's address*: Institut Ed. Van Beneden, quai Van Beneden 22, Liège, Belgium.

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par les données recueillies par électrophorèse, c'est probablement parce que les procédés électrophorétiques disponibles ne permettaient pas de déceler des fractions de très faible concentration ou de séparer des éléments de mobilité très voisine.

Cependant Kohn (1958) a décrit un nouveau milieu-support pour électrophorèse (l'acétate de cellulose) qui permet de séparer de minuscules échantillons de protéine (3–4 μl.) de manière très satisfaisante. Il m'a semblé intéressant de soumettre à l'électrophorèse sur ce nouveau milieu des extraits provenant d'un seul embryon et même de fragments d'embryon. Des essais préliminaires (Denis, 1960) ont montré que l'œuf d'Amphibien contient suffisamment de protéines pour fournir un spectre électrophorétique clair sur acétate de cellulose.

MATÉRIEL ET MÉTHODES

Les expériences ont porté sur l'Urodèle *Pleurodeles waltii*. Deux pontes seulement ont été utilisées: l'une a fourni les embryons pour les extraits totaux; l'autre a servi pour le prélèvement des fragments d'embryon. De la sorte, la variabilité de ponte à ponte se trouve éliminée dans chaque série d'expériences. Les stades ont été déterminés d'après la table de Gallien & Durocher (1957).

Isolement du matériel

Après déganguage et rinçage, les embryons de divers stades (0–37) sont transférés dans une solution saline (NaCl 0,65 pour cent) ajustée à pH 8,6 par l'addition de tampon véronal 0,02 M.

Chaque embryon est immédiatement aspiré à l'intérieur d'un tube capillaire (diamètre intérieur 1 mm.) dans $\pm$ 5μl. de solution saline. Le tube est ensuite scellé à la flamme d'un côté, bouché et conservé à la glacière ($-$ 20° C.) jusqu'au moment de l'utilisation. Un seul embryon sert pour chaque électrophorèse.

Pour le prélèvement de fragments de tissu, six embryons de même stade sont utilisés. L'opération est accomplie en solution de Holtfreter normale refroidie dans un bain de glace. Cette précaution facilite grandement la séparation des couches cellulaires du germe et d'autre part permet de conserver les embryons rigoureusement au même stade pendant toute la durée de l'opération.

Les tissus suivants ont été isolés et débarrassés soigneusement de toute cellule étrangère au moyen d'une boucle de platine: au stade blastula (6–7), ectoderme animal et endoderme; au stade jeune gastrula (8–9), ectoderme animal, endoderme et lèvre dorsale du blastopore; au stade gastrula âgée et neurula (11–15), toute la plaque neurale, le toit de l'archentéron, ectoderme ventral et endoderme; aux stades ultérieurs (15–27), le tube neural, l'axe chordo-somitique, ectoderme ventral et endoderme.

Les fragments de tissu provenant des six embryons opérés en même temps sont ensuite transférés en solution saline (NaCl 0,65 pour cent) rincés trois fois dans celle-ci et enfin aspirés dans 5 μl. de liquide. La conservation est assurée par congélation à $-$20° C., comme pour les embryons entiers.

Extraction

Le matériel mis en réserve est dégelé juste avant l'emploi. Les embryons et les fragments de tissu sont broyés soigneusement à l'intérieur de leur tube à l'aide d'un minuscule pilon de verre. La largeur de celui-ci correspond exactement au diamètre intérieur du capillaire, de sorte que le matériel se trouve rapidement réduit en une pulpe de couleur grisâtre.

Les homogénats sont ensuite centrifugés à 10.000 g pendant 20 minutes. Le culot de centrifugation est éliminé et seul le surnageant limpide est utilisé pour l'électrophorèse.

Électrophorèse

L'électrophorèse est conduite dans une solution de tampon véronal de pH 8,6 et force ionique $\mu = 0{,}05$. Chaque extrait est appliqué rapidement à l'aide du tube capillaire qui le contient sur une bande d'acétate de cellulose de 10×5 cm. La ligne de départ (3,5 cm. de long et 1 mm. de large) se trouve à égale distance du pôle positif et du pôle négatif. Une goutte de sérum de coq est aussi déposée sur la ligne de départ. L'albumine et les globulines sériques serviront comme référence pour l'étude de la mobilité des protéines embryonnaires. Un courant d'intensité constante (1,25 mA. par bande) est alors appliqué pendant une heure à raison de 10 volts par cm. Quatre bandes sont soumises à l'électrophorèse en même temps.

Coloration

Une fois l'électrophorèse terminée, les feuilles sont plongées, sans séchage préalable, dans une solution très diluée de nigrosine (1/20.000), contenant 3 pour cent d'acide trichloracétique (fixateur). Après 18 heures, la coloration atteint son maximum. Les feuilles sont alors rincées puis séchées.

Étude quantitative

En vue d'estimer la quantité de protéine présente sur chacune des bandes d'électrophorèse, celles-ci ont été examinées au spectrophotomètre en lumière visible (jaune). Avant l'examen, les feuilles sont éclaircies à l'huile de paraffine et pressées entre deux lames de verre. L'extinction est mesurée mm. par mm. à l'aide d'un pinceau lumineux de 1 mm. de large et de 15 mm. de long. Les valeurs lues sont portées en graphique où figurent en ordonnée les mesures d'extinction et en abscisse les distances de la ligne de départ. La surface comprise entre l'axe des abscisses et la courbe d'extinction donne une mesure (conventionnelle) de la quantité de protéines présente sur la feuille d'électrophorèse. Pour chaque protéinogramme, cette surface a été estimée par pesée.

RÉSULTATS

Extraits totaux

Résultats qualitatifs

Environ 110 électrophorèses (2 à 5 par stade) portant sur des extraits d'embryons entiers ont été menées à bien. Les résultats sont résumés qualitativement en un graphique (fig. 1) portant en ordonnée les mobilités électrophorétiques (en cm. par heure) et en abscisse la racine carrée du temps de développement à 18° C. On peut ainsi suivre facilement le sort des bandes électrophorétiques

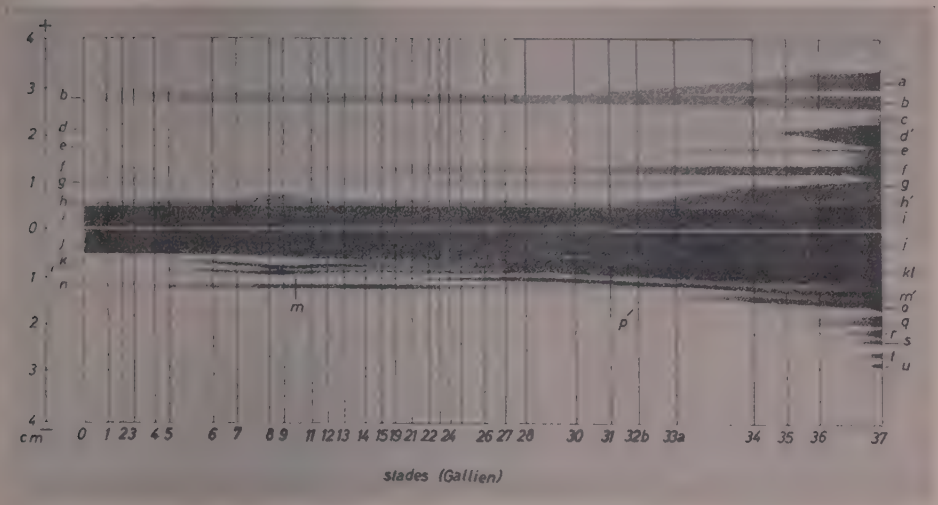

FIG. 1. Évolution du spectre électrophorétique au cours du développement. La ligne d'abscisse est graduée en $\sqrt{t}$.

durant toute l'ontogèneese. L'échelle des abscisses a été graduée en $\sqrt{t}$ afin de réduire l'espacement entre les stades avancés (30 à 37). En effet, les stades larvaires âgés sont difficiles à distinguer les uns des autres et ne correspondent pas toujours aux images données par la table de développement. Les conséquences de confusions possibles sont atténuées par le rapprochement, sur le diagramme, des points représentant les dernières étapes de l'ontogenèse.

Nomenclature. Les bandes protéiques sont désignées par les lettres de l'alphabet en commençant par les plus rapides (les plus proches du pôle positif). Dans cette nomenclature, il n'est pas tenu compte du moment d'apparition des diverses bandes. Lorsque l'intensité ou la mobilité d'un élément du spectre électrophorétique change de façon appréciable au cours du développement, la lettre qui le désigne est affectée d'une apostrophe.

Dans l'œuf indivis, 11 bandes d'intensité très différente peuvent être

distinguées: 7 du côté positif de la ligne de départ et 4 du côté négatif. Deux d'entre elles, très faibles (*d* et *e*), ne sont pas visibles dans tous les cas.

Toutefois, la répartition des bandes protéiques de chaque côté de la ligne de départ ne signifie nullement que les unes ont une charge positive et les autres une charge négative au pH étudié. Par suite de l'électroendosmose (Block, Durrum, & Zweig, 1958), toutes les bandes protéiques et la ligne de départ se trouvent décalées vers le pôle négatif à la fin de l'électrophorèse.

La ligne de départ réelle doit être placée légèrement en dessous de la bande *m* (−1,45 cm.). De la sorte, toutes les bandes du spectre apparaissent au stade 0 comme électronégatives.

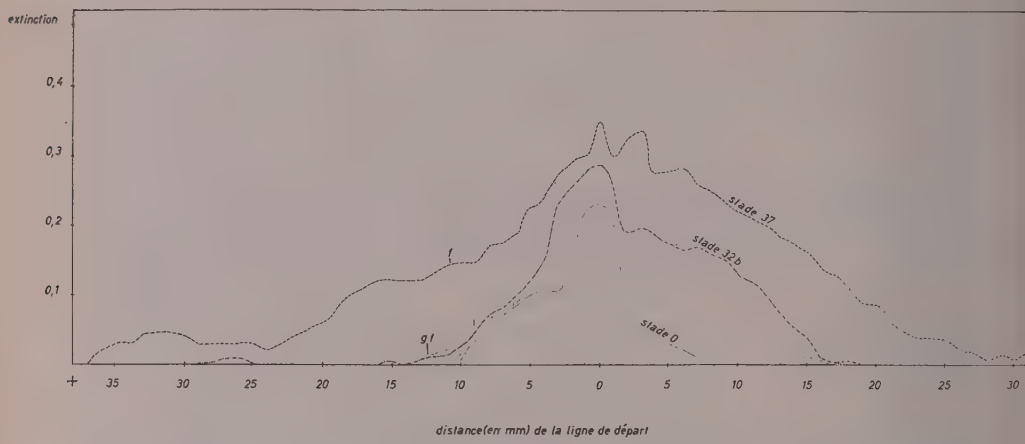

FIG. 2. Courbe d'extinction, de trois protéinogrammes à trois stades différents du développement.

Durant la segmentation, la bande *h* s'élargit et trois autres éléments (*k*, *l* et *n*) se renforcent. Au début de la gastrulation (stades 8–9), *k* et *l* s'intensifient de façon considérable et s'écartent légèrement de la ligne de départ. La bande *h* atteint à ce moment sa largeur maximum. Vers le stade 8, un élément de faible intensité (*m*) apparaît entre *l* et *n*.

Pendant le reste de la gastrulation et la neurulation, l'intensité de *k* et *l* diminue fortement; celle de *n* s'atténue aussi quelque peu. La largeur de *h* s'amenuise et redevient à peu près ce qu'elle était au cours de la segmentation. Entre les stades 12 et 15, la bande *m* se renforce de façon très notable.

Du stade 19 au stade 26, aucune transformation importante ne se produit. Seule est à noter une oblitération progressive de l'espace clair situé entre les bandes *j* et *k*. A partir du stade 26, la bande *m* se renforce et s'éloigne légèrement de la ligne de départ. Elle finit par recouvrir tout à fait la bande *n* qui s'affaiblit de plus en plus.

Au stade 33, la bande *b* commence à s'élargir. *E*, *f*, *i*, *j*, *k* et *l* se marquent

davantage. *H* augmente à la fois d'intensité et de largeur. Quatre nouvelles bandes (*o*, *p*, *q* et *r*) font leur apparition et ne tardent pas à se renforcer.

Au dernier stade examiné (37) la bande *b* s'est tout à fait dédoublée. La bande *d*, très faible jusque là, s'est élargie et intensifiée très fortement. Toutes les autres bandes voient leur intensité augmenter encore. Trois nouveaux éléments complètent le spectre du côté négatif: *s*, *t* et *u*.

Résultats quantitatifs

Au cours du développement, on constate une augmentation de la quantité de protéine extractible obtenue par embryon. Cette évolution est illustrée par la figure 2 où sont comparées les courbes d'extinction fournies par le protéinogramme de trois embryons à trois stades différents: stade 0 (0 heure;

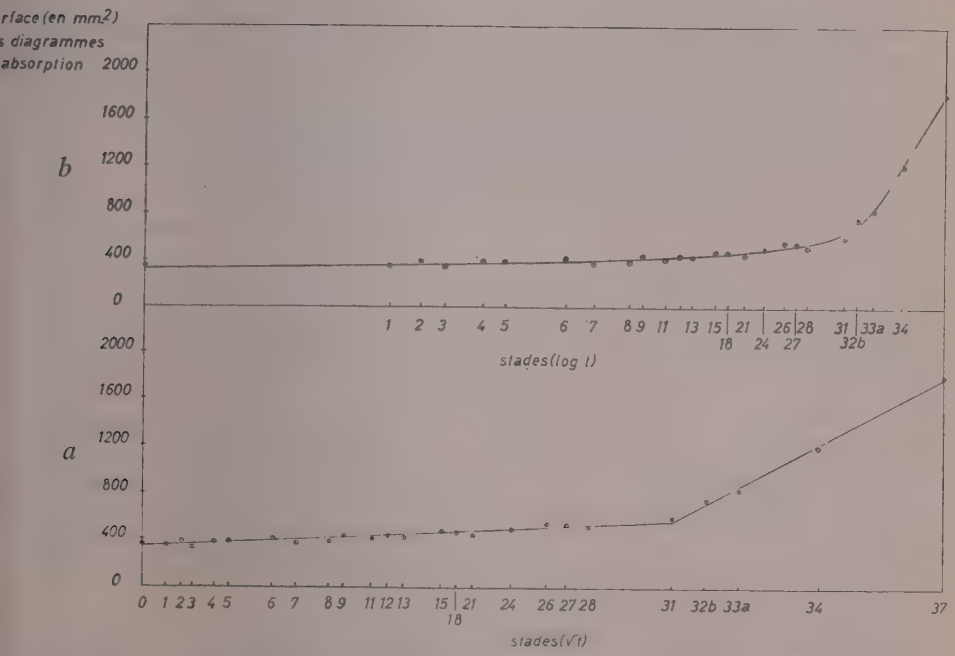

FIG. 3. Évolution de la quantité de protéines solubles par embryon entier au cours du développement. *a*, ligne d'abcisse graduée en $\sqrt{t}$; *b*, ligne d'abcisse graduée en log. *t*.

œuf fécondé mais indivis), stade 32*b* (190 heures) et stade 37 (360 heures; dernier stade avant la prise de nourriture).

Quelques remarques sont nécessaires à propos de ce diagramme.

Plusieurs bandes, visibles par transparence sur les feuilles d'électrophorèse (comme *b*, *d* et *e* du stade 0), sont d'intensité trop faible pour donner une extinction mesurable au spectrophotomètre. Elles ne sont donc pas présentes sur la figure.

Certaines bandes très proches (comme *f* et *g* du stade 0) sont confondues dans un seul pic d'absorption.

Des bandes très marquées (comme *f* du stade 37) se traduisent sur la courbe d'extinction, non par un pic très distinct, mais seulement par un palier ou même par une diminution de pente.

Ces particularités sont dues au type de spectrophotomètre utilisé et à la largeur relativement grande du pinceau lumineux par rapport aux bandes du spectre embryonnaire.

Les surfaces délimitées par la courbe d'extinction ont été mesurées et groupées par stade. Les valeurs moyennes sont portées en ordonnée sur un diagramme dont l'abscisse est graduée en racine carrée du temps de développement (fig. 3*a*). Les points obtenus se répartissent nettement en deux segments de droite. La première partie a une pente de 0,050, la seconde une pente de 0,536. Le point de rencontre des deux droites se trouve à hauteur du stade 31. Les stades de développement peuvent aussi être répartis sur la ligne d'abscisse en fonction de log de *t*. Les points obtenus se groupent alors sur une courbe d'allure exponentielle. Le point d'inflexion de celle-ci se situe aux environs du stade 31 (fig. 3*b*).

Extraits de fragments d'embryon

Huit cents fragments de tissu ont été prélevés sur environ 200 embryons et soumis à l'électrophorèse. Les résultats ne permettent qu'une analyse qualitative. En effet, la quantité de tissu isolée n'est pas exactement la même dans chaque prélèvement. Un examen au spectrophotomètre des protéinogrammes obtenus ne donnerait donc pas de résultats comparables. Les données recueillies ont été rassemblées en quatre diagrammes dont l'abscisse est graduée en fonction de *t*.

Nomenclature. Plusieurs bandes électrophorétiques obtenues à partir de fragments de tissu paraissent en tous points identiques à des éléments du spectre électrophorétique d'embryons entiers. Dans ce cas, la dénomination adoptée pour les protéinogrammes d'extraits totaux est conservée. Quelques bandes, cependant, semblent appartenir en propre aux tissus embryonnaires. Elles seront désignées de la façon suivante:

ec_1, ec_2, ec_3, etc., pour l'ectoderme compétent;
ne_1, ne_2, ne_3, etc., pour l'ectoderme neural;
ve_1, ve_2, ve_3, etc., pour l'ectoderme ventral;
en_1, en_2, en_3, etc., pour l'endoderme.

Ectoderme neural

L'ectoderme de la zone animale de la blastula (stade 6) présente 5 bandes cathodiques et 5 bandes anodiques (fig. 4). Trois de ces dernières sont de faible intensité (ec_1, ec_2 et ec_3). Entre le stade 6 et le début de la gastrulation, la bande *h* s'élargit et se renforce légèrement. *J* augmente également de largeur et vers le stade 7 recouvre la bande ec_1. Celle-ci n'est désormais plus visible. Juste avant la formation du blastopore (stade 8), deux bandes (*k* et *l*) apparaissent au niveau

de ec_3 et masquent complètement cette dernière. En même temps, une bande supplémentaire (*m*) s'intercale entre *l* et *n*.

La largeur de *h* se réduit peu à peu dans la suite de la gastrulation. Les bandes *k* et *l* diminuent d'intensité et cessent d'être distinctes l'une de l'autre. En même temps, elles se rapprochent de la ligne de départ. Vers le stade 11, deux bandes faiblement marquées apparaissent du côté positif (*d* et *e*).

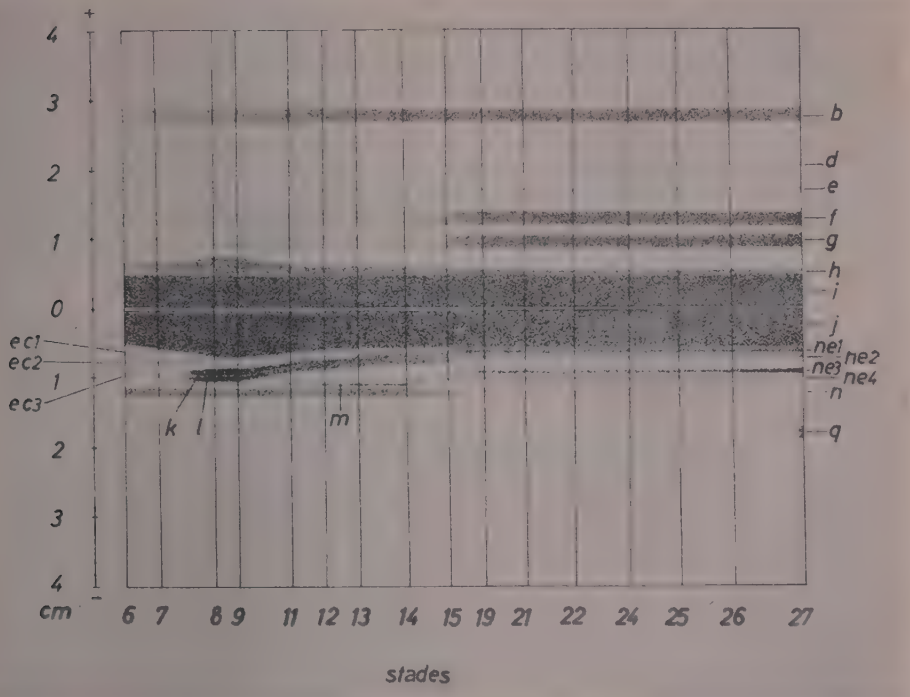

FIG. 4. Évolution du spectre électrophorétique de l'ectoderme neural. Ligne d'abscisse graduée en fonction de *t*.

Pendant la neurulation, l'intensité de *m* s'accroît et atteint son maximum au stade 14. Cette bande s'estompe un peu plus tard (stade 15). L'élément unique produit par la fusion de *k* et *l* continue à se rapprocher de la ligne de départ. Vers le stade 19, il fait place à deux bandes étroites et bien distinctes: ne_1 et ne_2. L'espace laissé vide par le retrait de *k* et *l* se garnit de deux nouveaux éléments: ne_3 et ne_4. Pendant la suite de la vie larvaire, ne_3 se renforce petit à petit. L'intensité de ne_4, par contre, demeure plus ou moins constante. De son côté, *n* se réduit progressivement jusqu'au stade 27. Au dernier stade étudié, une bande électronégative fait son apparition (*p*).

Entre le premier et le dernier stade examiné, la quantité totale de protéines fournies par l'ectoderme neural semble diminuer quelque peu.

Ectoderme ventral

Jusqu'au stade 10, l'évolution de l'ectoderme ventral est identique à celle de l'ectoderme neural. Dès la seconde partie de la neurulation, cette évolution devient divergente (fig. 5).

Les bandes *k* et *l* diminuent d'intensité et se rapprochent de la ligne de départ beaucoup plus rapidement que dans le tissu nerveux présomptif.

La bande *m* disparaît dès le stade 12. Dans l'ectoderme neural, la bande correspondante se maintient jusqu'au bout de la neurulation.

Vers le stade 12, une bande de faible intensité (ve_5) s'intercale entre *l* et *n*. Dans le tissu nerveux, aucun élément nouveau n'apparaît à ce moment.

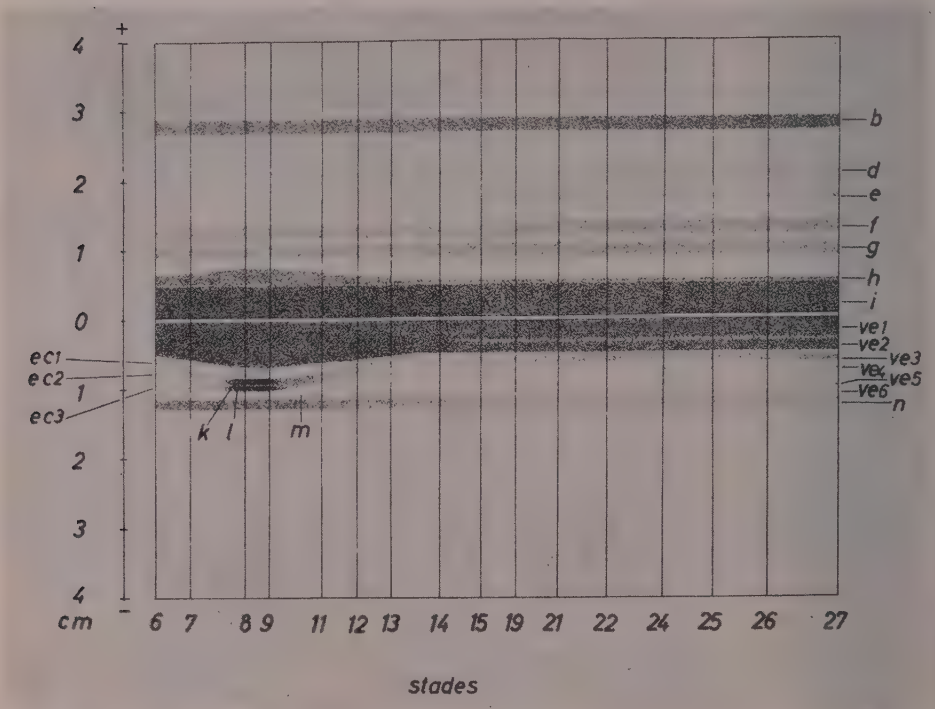

FIG. 5. Évolution du spectre électrophorétique de l'ectoderme ventral. Ligne d'abscisse graduée en fonction de *t*.

Durant la neurulation, les divergences entre ectoderme neural et ectoderme ventral s'accentuent:

La disparition presque complète de *k* et *l* laisse entre *j* et *n* un grand espace clair. Il n'existe pas de semblable hiatus dans le protéinogramme du tissu nerveux.

Au stade 19, la bande *j* se divise en deux parties (ve_1 et ve_2). Cette bande reste, au contraire, simple dans l'ectoderme neural.

Il n'existe qu'une seule bande dans le prolongement de *k* et *l* (ve_3). Le spectre fourni par l'ectoderme neural présente à ce niveau deux éléments distincts (ne_1 et ne_3).

Après la fin de la neurulation, deux bandes de faible intensité (ve_4 et ve_6) apparaissent de part et d'autre de ve_5. Par la suite, plus aucun changement important ne se manifeste jusqu'au stade 27.

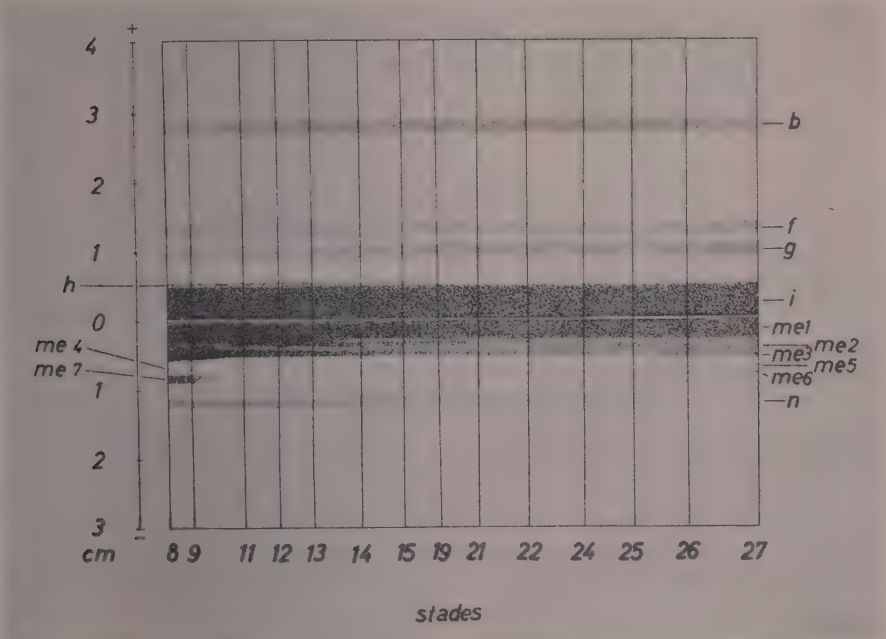

FIG. 6. Évolution du spectre électrophorétique du mésoderme. Ligne d'abscisse graduée en fonction de *t*.

Au dernier stade examiné, la partie positive du spectre de l'ectoderme ventral est très semblable à celle observée dans le tissu nerveux. Du côté négatif, au contraire, seule la bande *n* semble commune aux deux tissus. La bande *j* est simple dans l'ectoderme neural et double dans l'ectoderme ventral.

De toutes les autres composantes anodiques, seules ve_5 (ectoderme ventral) et ne_4 (ectoderme neural) ont la même mobilité (— 10 mm. par heure) et peuvent être comparées l'une à l'autre. Les bandes ne_1 et ne_2 observées dans le tissu nerveux présomptif ont une mobilité de —6,5 et —8 mm. par heure et diffèrent des bandes ve_3 et ve_4 de l'ectoderme ventral. Celles-ci ont, en effet, une mobilité de — 6 et —7,5 mm. par heure. La bande ne_3, très marquée dans le tissu nerveux,

est absente dans l'ectoderme ventral. Il en va de même pour *p*. Par ailleurs, l'ectoderme ventral possède entre ve_5 et *n* une bande (ve_6) qui n'existe pas dans l'ectoderme neural.

Mésoderme

Au premier stade (8) examiné, le spectre électrophorétique du mésoderme présente 4 bandes du côté positif de la ligne de départ et 5 du côté négatif (fig. 6). Toute la partie cathodique du protéinogramme est moins marquée que dans l'ectoderme. La bande *h*, assez intense dans le tissu ectodermique, fait ici presque totalement défaut. Entre *j* et *n* se placent deux éléments (me_4 et me_7) d'intensité beaucoup plus faible que les bandes ectodermiques *k* et *l*. Me_4 et me_7 semblent distinctes de *k* et *l*. En effet, la mobilité de me_4 et me_7 d'une part, et de *k* et *l* d'autre part ne correspond pas. Me_4 et me_7 se réduisent progressivement dans la suite de la gastrulation. Me_4 disparaît tout à fait après le stade 11.

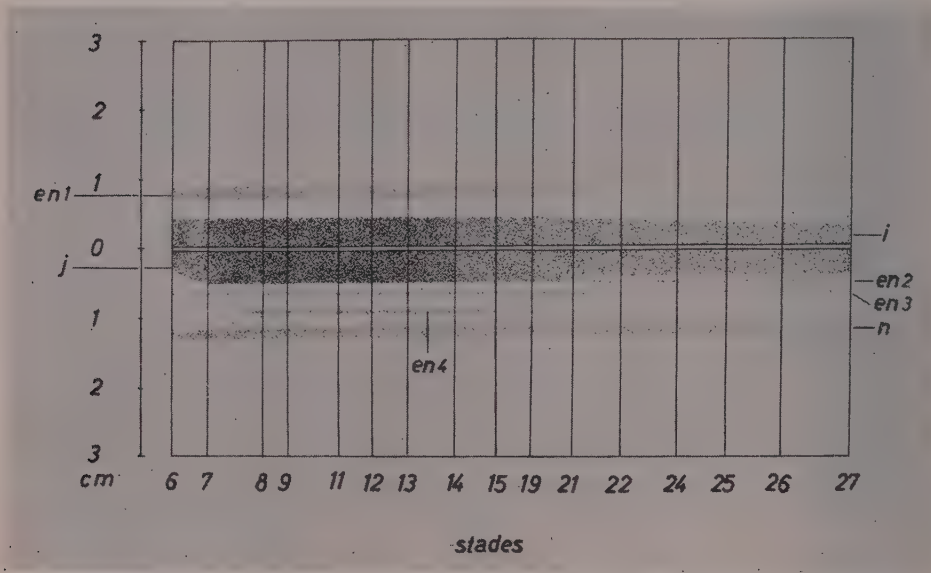

FIG. 7. Évolution du spectre électrophorétique de l'endoderme. Ligne d'abscisse graduée en fonction de *t*.

Au stade 13, la bande *j* se divise en trois parties (me_1, me_2 et me_3). Deux nouvelles bandes faiblement marquées (me_5 et me_6) apparaissent entre *j* et me_7. Durant la neurulation et le début de la vie larvaire, plus aucune modification importante ne se produit.

Au dernier stade examiné, le spectre électrophorétique donné par le mésoderme se montre donc notablement moins complexe que celui fourni par l'ectoderme. Du côté positif, les bandes ectodermiques *d*, *e* et *h* sont absentes.

Du côté négatif, seule la bande *n* paraît commune à l'ectoderme et au mésoderme. La bande *j*, simple dans le tissu nerveux présomptif et double dans l'ectoderme ventral, est ici composée de trois éléments distincts.

Quant au groupe de trois bandes me_5, me_6 et me_7, leur mobilité ne correspond pas à celle des bandes ne_1, ne_2 et ne_3 de l'ectoderme neural et des bandes ve_3, ve_4 et ve_5 de l'ectoderme ventral.

Endoderme

Le spectre électrophorétique fourni par le tissu endodermique est beaucoup plus pâle et moins complet que celui de l'ectoderme et du mésoderme (fig. 7). Il ne comprend que deux bandes cathodiques: en_1 et *i*. Le plus rapide de ces deux éléments (en_1) disparaît du reste entre le stade 20 et 26. La bande *i* a une largeur moindre (4 mm.) que la bande correspondante de l'ectoderme et du mésoderme. De plus, elle se rétrécit encore au cours du développement (3,5 mm. au stade 27).

Du côté négatif de la ligne de départ, il n'existe au stade 6 que trois éléments: *j*, en_3 et *n*. Au début de la gastrulation, une nouvelle bande (en_4) apparaît entre en_3 et *n*. Cette bande s'affaiblit après le stade 20 et disparaît tout à fait. Après la fin de la neurulation, *j*, en_3 et *n* diminuent également d'intensité. Au dernier stade examiné, *n* s'est presque totalement effacée. Il faut encore noter au début de la vie larvaire la division de *j* en deux parties d'importance inégale.

DISCUSSION DES RÉSULTATS

Valeur des résultats quantitatifs

L'estimation de la quantité de protéines présente sur chaque feuille d'électrophorèse a été influencée par différents facteurs.

1. Tout le volume de l'extrait peut n'avoir pas été appliqué sur la bande d'acétate de cellulose. Cette source de variation a dû intervenir dans certains cas. Il est, en effet, difficile de séparer de façon parfaite le surnageant du résidu insoluble après centrifugation d'un seul embryon. Toutefois, cette erreur expérimentale ne doit pas avoir influencé les résultats dans un sens déterminé: elle a pu intervenir avec une égale fréquence à tous les stades étudiés.

2. L'intensité de la coloration peut différer d'une expérience à l'autre. La nigrosine, colorant très sensible, pâlit assez rapidement. Son pouvoir colorant peut varier d'un jour à l'autre et même au cours d'une seule coloration. Certains groupes de feuilles traitées en même temps seront donc plus colorées que d'autres. Toutefois, les individus soumis à une même électrophorèse appartiennent à des stades répartis au hasard dans toute la série des étapes de développement. La seconde cause de variation agit donc sur toute la gamme des stades et ne paraît pas avoir influencé certains résultats de façon préférentielle.

3. Selon Jenks, Setton, & Durrum (1955), l'intensité de la coloration d'une protéine séparée par électrophorèse sur papier n'est pas toujours proportionnelle à sa concentration. Cette discordance résulte du fait que tous les colorants

ne suivent pas la loi de Lambert-Beer. La concentration relative des protéines les plus abondantes peut donc être sous-estimée. Il semble cependant que cette cause d'erreur affecte surtout les protéines (telles que l'albumine sérique) présentes sur la feuille d'électrophorèse à très forte concentration. Dans nos expériences, la quantité de protéines appliquée sur chaque bande d'électrophorèse n'est jamais très forte. La source d'erreur précitée ne paraît pas avoir influencé les résultats de façon notable.

Plusieurs auteurs ont noté la variabilité considérable qui peut affecter les résultats expérimentaux obtenus à partir de matériel embryonnaire. Ce manque d'homogénéité est surtout important lorsqu'il s'agit du dosage de constituants chimiques. La principale cause de variation est constituée par les différences de ponte à ponte. Les expériences présentes ont été effectuées sur un seul lot d'œufs. Tout porte à croire que nous avons affaire à une population relativement homogène obéissant aux lois de distribution normale.

Étude statistique. Au cours du développement la quantité totale de protéines solubles fournie par un embryon s'accroît de façon régulière. Cette augmentation est-elle statistiquement significative? Pour le vérifier, les résultats ont été soumis à l'analyse de variance.

Il a été nécessaire de grouper les données relatives à la première partie du développement (0–28). En effet,

1. Les stades embryonnaires précoces sont peu espacés dans le temps. Aucune différence ne se manifeste quand on compare deux stade successifs.
2. Considérés par étape de développement isolée, les résultats sont sujets à une variabilité importante. Des différences significatives n'apparaissent qu'entre des stades très éloignés l'un de l'autre (0 et 14 par exemple).

Le groupement a été réalisé de la façon suivante (tableau 1).

TABLEAU 1

Stades	*Groupe*	*Dénomination*	*Durée moyenne de développement* (18° C.)
0, 1, 2, 3	A	Segmentation 1ère partie	5 h.
4, 5, 6, 7, 8	B	Segmentation 2ème partie	23 h. 30
9, 11, 12	C	Gastrulation	43 h. 15
13, 15, 18, 21	D	Neurulation	72 h.
24, 26, 27, 28	E	Bourgeon caudal	114 h. 30
31	F	Mise en marche de la circulation	172 h. 30
32 b	G		190 h.
33 a	H		213 h.
34	I		264 h.
37	J	Dernier stade avant la prise de nourriture	360 h.

Huit comparaisons ont été effectués (tableau 2). Malgré les diverses sources de variation discutées plus haut, il apparaît une augmentation significative de la quantité de protéines solubles par embryon:

entre le début de la segmentation et la gastrulation,
entre la deuxième partie de la segmentation et la neurulation,
entre la gastrulation et la formation du bourgeon caudal,
entre la neurulation et la mise en marche de la circulation,
entre les différents stades postérieurs à la mise en marche de la circulation.

La fig. 3*a* montre que la vitesse d'apparition des protéines solubles augmente brusquement vers le stade 31. A quel moment exact faut-il situer cette accélération? Devons-nous bien placer au niveau du stade 31 l'intersection des deux segments de droite visibles sur la figure 3*a*? Le premier point de ce diagramme qui se trouve hors du prolongement du segment de droite initial correspond au stade 32*b*. Si les protéines solubles apparaissaient après le stade 31 au même rythme qu'avant celui-ci, la mesure de la quantité totale de protéines au stade 32*b* devrait être, avec la technique utilisée, de 600 mm². Pour les mesures faites à ce dernier point, l'intervalle de confiance de la moyenne s'étend de 610,4 à 880 mm², au niveau de la probabilité de 95 pour cent. En d'autres termes, la probabilité que la moyenne réelle des mesures effectuées au point 32*b* ait une valeur inférieure à 610,4 est de 2,5 pour cent. Nous avons donc de bonnes raisons de croire que la moyenne réelle au point 32*b* se trouve au-dessus du segment de droite 0–31. L'accélération visible sur la figure 3*a* se situe très probablement entre les stades 31 (172 h. 30) et 32*b* (190 h.).

Le spectre électrophorétique au cours de la segmentation

Plusieurs bandes se renforcent visiblement pendant la période du clivage: *k*, *l* et *n*. La fig. 3*a* semble indiquer par ailleurs un accroissement de la quantité totale de protéines dans l'œuf en segmentation. Toutefois, l'augmentation observée entre les stades 0 et 7 n'est pas statistiquement significative: le nombre d'expériences effectuées à ces deux stades est trop faible et les résultats obtenus présentent une variabilité trop grande.

TABLEAU 2

Comparaison		*Rapport de variance* F
1	A–C	5,10 significatif*
2	B–D	5,43 „
3	C–E	8,39 hautement significatif
4	D–F	13,84 „
5	E–G	33,11 „
6	F–H	22,90 „
7	G–I	77,01 „
8	I–J	76,48 „

* $F_{0,05}$: 3,95. $F_{0,01}$: 6,97.

Entre la 5ème et la 43ème heure de développement, la quantité totale de protéines par embryon s'accroît de façon significative (tableau 2). Si la quantité globale de protéines solubles restait stationnaire durant le clivage, toute l'augmentation (18,4 pour cent) observée entre les groupes de comparaison A et C devrait se

produire dans le court intervalle de temps qui sépare l'apparition du blastopore (31 h.) du milieu de la gastrulation (43 h.). Cette hypothèse est peu vraisemblable. L'allure des courbes 3*a* et 3*b* semble au contraire indiquer que l'accroissement se répartit sur toute la durée qui sépare le stade 0 du stade 12. L'apparition de nouvelles quantités de protéines solubles commencerait donc dans l'œuf dès les premières segmentations. Le renforcement très visible de plusieurs bandes électrophorétiques à partir du stade 0 confirme cette supposition.

La segmentation des Amphibiens est généralement considérée comme une période relativement inerte au point de vue des transformations chimiques. Avant le stade 18, Gregg & Ballentine (1946) ne détectent aucun accroissement de la quantité d'azote extractible par embryon (*Rana pipiens*). D'après Hoff-Jørgensen & Zeuthen (1952) il n'y a pas de synthèse d'ADN ni d'anabolisme protéique durant les 16 premières heures de développement (*R. platyrrhina*). De même Løvtrup (1955) n'observe aucun accroissement de la quantité d'acide nucléique et de l'activité enzymatique pendant la période du clivage.

Nos résultats sont partiellement en accord avec les données précédentes. Dans nos expériences, en effet, aucune nouvelle espèce de protéine n'apparaît durant la segmentation. Par contre, la quantité totale de protéines extractibles semble s'accroître régulièrement au cours de cette même période. Cette augmentation n'est peut-être pas due à un processus de synthèse. Il s'agirait plutôt de la libération de certaines protéines du vitellus à l'intervention d'une phosphoprotéinephosphatase (PPPase). Selon Barth & Barth (1951) et Mezger-Freed (1953), il existe une notable activité PPPasique durant le clivage. L'utilisation des réserves vitellines commencerait donc dès le début de l'ontogenèse. Le renforcement des bandes *k*, *l* et *n* pourrait résulter d'un apport direct à partir du matériel vitellin.

Le spectre électrophorétique durant la période s'étendant de la gastrulation au stade 31

Le début de la gastrulation est marqué par le renforcement très notable des bandes *h*, *k* et *l*. De plus, le spectre électrophorétique s'enrichit d'un nouvel élément: *m*.

De nombreux faits tirés de la littérature suggèrent que la mise en marche des synthèses protéiques coïncide avec le début de la gastrulation.

Clayton (1953) note à ce moment l'apparition d'un nouvel antigène. Kutsky (1950) observe une augmentation de la quantité d'acides nucléiques au moment de la formation du blastopore. Un tel phénomène est sans doute en relation avec le démarrage des mécanismes de synthèse. Dans nos expériences, l'apparition de la bande *m* constitue vraisemblablement la première manifestation de l'anabolisme protéique de l'embryon.

La quantité totale de protéines extractibles par embryon s'accroît lentement du début à la fin de la gastrulation (fig. 3*a*). Cette augmentation est sans doute le

reflet de l'apparition de nouvelles quantités de cytoplasme actif. Les protéines nécessaires à l'accroissement du cytoplasme proviennent vraisemblablement d'une digestion progressive du matériel vitellin. Ce phénomène semble bien en cours depuis le début de l'ontogenèse. Selon Barth & Barth (1951) et Mezger-Freed (1953), l'attaque enzymatique du vitellus se poursuit à un rythme élevé durant la gastrulation.

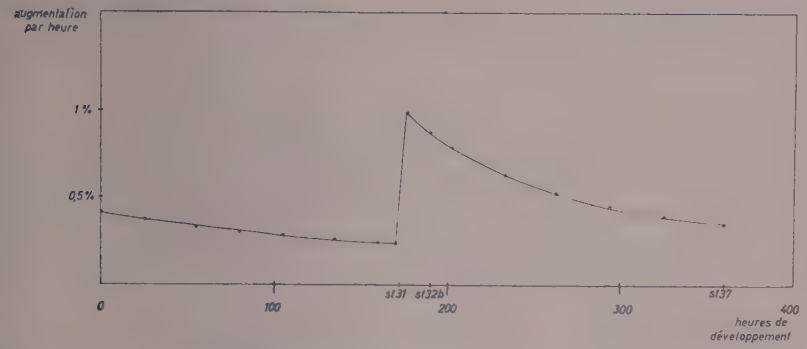

FIG. 8. Évolution de l'accroissement horaire de la quantité de protéines solubles ramenée à la quantité totale de protéines solubles.

L'augmentation lente de la quantité totale de protéines solubles au cours de la gastrulation résulte surtout du renforcement de plusieurs bandes électrophorétiques (principalement *i* et *j*). En regard de cette intensification, l'apport représenté par la nouvelle bande *m* semble tout à fait insignifiant. Dans l'augmentation générale de la quantité de protéines solubles, la part fournie par les éléments protéiques nouveaux paraît donc assez minime. Cette situation semble se maintenir durant toute la période qui s'étend du stade 9 au stade 31 : aucune bande protéique nouvelle n'apparaît durant cet intervalle de temps sur les protéinogrammes d'embryons entiers.

Le spectre électrophorétique durant la période postérieure au stade 31

Entre le stade 31 et la fin de l'ontogenèse, 9 nouvelles bandes protéiques font leur apparition. Le spectre électrophorétique qui, de la fécondation au stade 31, avait assez peu évolué, se transforme et se diversifie rapidement. Plusieurs organes (cœur, somites) deviennent à présent fonctionnels. Cette mise en fonction implique la synthèse de protéines spécifiques (telles que hémoglobine, sérum-globulines, myoglobuline, etc.). Les nouvelles bandes qui apparaissent après le stade 31 sont sans doute une illustration de ce phénomène.

Cette diversification rapide de l'arsenal protéique se marque par une accélération dans la vitesse d'apparition des protéines solubles. Cette accélération se situe entre les stades 31 et 32*b* (voir page 435). A partir du stade 31, l'augmentation de la quantité de protéines extractibles se poursuit à un rythme

accéléré mais constant. Chaque heure de développement voit les protéines solubles s'accroître d'une même quantité. Cette vitesse d'accroissement est 4,25 fois supérieure à celle qui prévalait dans la première partie de l'ontogenèse (stades 0–31).

Si l'augmentation horaire de la quantité de protéines extractibles est ramenée à la quantité totale de protéines solubles par embryon, la courbe d'accroissement prend une allure très caractéristique (fig. 8). Sous cette forme, le diagramme présente une cassure très nette entre les stades 31 et 32*b*.

La brusque accélération observée sur la fig. 8 dans la vitesse d'apparition des protéines extractibles résulte probablement de la mise en marche de la circulation sanguine. Ce phénomène intervient aux environs du stade 31.

Jusqu'au stade 31, la masse vitelline endodermique de l'embryon reste relativement intacte. Ce n'est que vers la 200ème heure de développement (stade 32–33) que le vitellus endodermique commence à être résorbé de façon visible. Pourtant, une activité enzymatique paraît déjà exister dans l'endoderme avant le stade 31. Panijel (1950), Gross (1952) et Flickinger (1956) ont montré qu'une activité PPPasique intense se trouve liée aux grains de vitellus au cours des stades précoces de l'ontogenèse. Cette activité est même considérable dans le vitellus végétatif (Barth & Barth, 1951). Un équilibre enzymatique existerait donc depuis le début du développement au sein de la masse vitelline endodermique entre les protéines insolubles du vitellus et les protéines solubles libérées par la PPPase. Mais cet équilibre est fortement déplacé en faveur des protéines insolubles. Jusqu'au stade 27, le tissu endodermique contient très peu de protéines extractibles (voir page 433).

L'ébranlement des fluides internes suite à la mise en marche de la circulation semble devoir modifier profondément l'équilibre enzymatique au sein de l'endoderme. Le lavage continuel de la surface externe du futur intestin par le liquide circulatoire emporterait les protéines solubles libérées par la PPPase endodermique. Cette évacuation entraînerait de proche en proche la libération de nouvelles quantités de protéines. Celles-ci peuvent être soit utilisées comme telles, soit dégradées plus complètement avant de servir dans l'élaboration de molécules protéiques entièrement nouvelles. Quoi qu'il en soit, la mise en marche de la circulation et la digestion accélérée du vitellus qui en résulte, doivent être la cause directe ou indirecte de l'augmentation observée vers le stade 31 dans la vitesse d'apparition des protéines solubles.

Le spectre électrophorétique des fragments de tissu

L'intensité du spectre électrophorétique au cours du développement

Les protéinogrammes fournis par les différents tissus embryonnaires (ectoderme neural, ectoderme ventral, mésoderme et endoderme) semblent diminuer d'intensité après la fin de la neurulation (figs. 4, 5, 6 et 7). Au cours du développement, la quantité de protéines extractibles déclinerait donc à l'intérieur

de ces tissus. Cette diminution n'est qu'apparente. Elle provient des conditions expérimentales. Avant la fermeture du tube nerveux, le prélèvement d'échantillons de tissu pour l'électrophorèse est relativement aisé. Après le stade 20, les couches tissulaires adhèrent étroitement l'une à l'autre et les morceaux isolés sont toujours assez petits. La quantité de tissu soumise à l'extraction est donc plus faible quand elle provient d'embryons ayant achevé leur neurulation. Ainsi s'explique le pâlissement du spectre électrophorétique observé après le stade 20.

Existence de gradients dans la jeune gastrula

Dès les premiers stades examinés, les trois feuillets embryonnaires montrent un contenu protéique différent. Chaque tissu possède des bandes protéiques qui lui sont propres: *k* et *l* pour l'ectoderme, me_4 et me_7 pour le mésoderme, en_1 et en_3 pour l'endoderme. Certaines bandes sont par ailleurs communes à l'ectoderme et au mésoderme: *b*, *f*, *g*, *i* et *n*. D'autres éléments se retrouvent à la fois dans l'ectoderme, le mésoderme et l'endoderme: *i*, *j* et *n*.

Les données précédentes peuvent s'interpréter comme une preuve de l'existence d'un gradient animal-végétatif dans la répartition des protéines de la jeune gastrula. Ce gradient est à la fois quantitatif et qualitatif. En effet:

La concentration en protéines extractibles se montre beaucoup plus élevée dans le tissu animal que dans le tissu vitellin (figs. 4 et 7).

Certaines protéines n'existent que dans la partie animale de l'embryon. D'autres sont localisées uniquement dans la partie végétative.

L'absence de données comparatives entre les régions marginales dorsale et ventrale ne nous permet pas d'affirmer l'existence d'un gradient dorso-ventral. Toutefois, la concentration en protéines extractibles semble au moins aussi élevée dans le tissu mésodermique que dans le tissu ectodermique (figs. 4 et 6). Le spectre électrophorétique fourni par le tissu mésodermique est, en effet, d'intensité à peu près égale à celui que donne l'ectoderme. Il est vrai que les quantités de tissu prélevées pour l'étude électrophorétique de l'ectoderme et du mésoderme ne sont peut-être pas égales. Toutefois, les fragments de lèvre dorsale du blastopore examinés pour leur contenu protéique n'étaient jamais de taille supérieure aux fragments d'ectoderme animal. Il semble donc bien que, au début de la gastrulation, la concentration des protéines extractibles dans le mésoderme et dans l'ectoderme présomptifs soit comparable. La zone animale de haute teneur en protéines solubles se prolongerait par conséquent sous l'équateur de la gastrula dans la zone marginale dorsale. Au surplus, cette dernière région se montre qualitativement différente des parties plus animales (ectodermiques) et plus végétatives (endodermiques).

L'existence d'un gradient animal-végétatif dans la répartition des protéines est en accord avec la théorie de Dalcq & Pasteels (1937). Nos résultats sont également à rapprocher des données obtenues par Brachet (1940) concernant la répartition dans l'embryon des protéines à fonctions SH. Celles-ci se montrent

nettement plus abondantes dans la région animale de la blastula que dans la région végétative. Au début de la gastrulation, la lèvre dorsale du blastopore présente également une teneur élevée en protéines soufrées.

Évolution de l'ectoderme au cours de la gastrulation et de la neurulation

Entre le stade 6 et le stade 8, le spectre électrophorétique de l'ectoderme acquiert deux nouvelles bandes: *j* et *k*. Très marqués aux stades 8 et 9, ces éléments s'estompent petit à petit dans la seconde partie de la gastrulation. Cette disparition est beaucoup plus rapide dans l'ectoderme ventral que dans le tissu nerveux présomptif. La présence des bandes *j* et *k* dans l'ectoderme coïncide donc exactement avec la période de 'compétence' de celui-ci. En effet, la faculté du tissu ectodermique de se transformer en tissu nerveux est maximum au début de la gastrulation, et s'amenuise petit à petit dans la suite du développement. Il est permis de se demander si la compétence de l'ectoderme n'est pas en relation directe avec la présence au sein de celui-ci des bandes *j* et *k*. Ces protéines pourraient par exemple conférer au tissu ectodermique une perméabilité particulière le rendant sensible à la pénétration d'une substance inductrice.

Vers le stade 8, l'ectoderme se garnit d'une bande supplémentaire (*m*). L'évolution de ce nouvel élément est différente dans l'ectoderme neural et dans l'ectoderme ventral. Dans le tissu nerveux présomptif, la bande *m* se renforce au début de la période d'induction et se maintient jusqu'aux environs du stade 15. Dans l'ectoderme ventral au contraire, cet élément disparaît dès le stade 11. Le renforcement de la bande *m* dans le tissu nerveux présomptif pourrait être une conséquence de l'induction neurale.

Différenciation précoce des différents tissus

Entre le stade 14 et le stade 19, le spectre électrophorétique de l'ectoderme neural, de l'ectoderme ventral et du mésoderme s'enrichit de plusieurs bandes supplémentaires (ne_3 et ne_4 pour l'ectoderme neural, ve_4, ve_5 et ve_6 pour l'ectoderme ventral, me_5 et me_6 pour le mésoderme). Dès la fin de la neurulation (stade 20), il existe d'importantes différences dans la constitution protéique de l'ectoderme neural et de l'ectoderme ventral. Or, à ce moment, ces tissus sont déterminés de façon définitive.

Comparaison des données électrophorétiques fournies par les extraits d'embryons entiers et par les fragments de tissu

Certaines discordances apparaissent quand on compare le spectre électrophorétique d'embryons entiers et les protéinogrammes fournis par les différents tissus embryonnaires.

1. Dans le tissu ectodermique, les bandes *k* et *l* semblent apparaître au stade 8. Le spectre électrophorétique d'extraits totaux indique, au contraire, que ces bandes existent depuis le début du développement.

2. La bande *m* disparaît dans l'ectoderme neural vers le stade 15. Sur le protéinogramme 'total', cet élément paraît se maintenir jusqu'à la fin de l'ontogenèse.

Ces discordances peuvent cependant s'expliquer. Le spectre électrophorétique 'total' peut être considéré comme la superposition des protéinogrammes fournis par les différents tissus embryonnaires. Cependant, toutes les parties de l'embryon n'ont pas été étudiées au point de vue de leur contenu protéique. La région marginale ventrale, par exemple, a été laissée de côté. L'extrait 'total' contient donc des protéines provenant d'une plus grande variété de tissus que les quatre espèces étudiées.

Certaines bandes visibles sur le protéinogramme 'total', proviennent donc de parties d'embryon qui n'ont pas été examinées par électrophorèse. D'autres éléments — simples en apparence — du spectre électrophorétique 'total' résultent de la superposition de plusieurs bandes étroites fournies par des régions distinctes de l'embryon.

Ainsi en va-t-il probablement des bandes *k* et *l* pendant la période du clivage. Les bandes ec_2 et ec_3 de l'ectoderme, me_4 et me_7 du mésoderme, et en_3 de l'endoderme se superposent sans doute dans le protéinogramme 'total' et prennent l'aspect de deux bandes distinctes: *k* et *l*.

Quant à la bande *m*, visible sur le protéinogramme 'total', elle ne constitue probablement pas un élément simple mais double. Jusqu'au stade 15, elle correspondrait à la bande ectodermique de même nom. A partir du stade 19, elle serait produite par la superposition des bandes ve_6, ne_4 et peut-être ne_3. Celles-ci sont apparues dans l'ectoderme ventral et dans l'ectoderme neural au cours de la neurulation.

Le spectre électrophorétique de fragments de tissu met en évidence certains faits qui n'apparaissent pas à l'examen du protéinogramme 'total'. Ainsi, l'ectoderme et le mésoderme sont le siège d'une synthèse importante de matériel protéique au cours de la neurulation. Toutes les nouvelles bandes visibles dans les tissus isolés se recouvrent l'une l'autre dans le protéinogramme 'total' et passent inaperçues.

CONCLUSIONS GÉNÉRALES

Au point de vue du métabolisme protéique, le développement peut être divisé en trois phases principales:

1ère phase: de la fécondation au début de la gastrulation (clivage).

2ème phase: du début de la gastrulation au stade 31.

3ème phase: du stade 31 à la prise de nourriture.

La première période est caractérisée par un accroissement lent de la quantité totale de protéines extractibles. Cette augmentation n'est probablement pas due à des synthèses protéiques mais à une mise en solution progressive des réserves vitellines.

Au cours de la seconde période, l'anabolisme protéique se met en marche. En effet, une nouvelle bande apparaît au stade 8 et plusieurs autres au cours de la neurulation. Malgré cela, la vitesse d'apparition des protéines solubles demeure sensiblement égale à ce qu'elle était durant le clivage.

Vers le stade 31, la vitesse d'apparition des protéines solubles augmente brusquement. En même temps, se produit une diversification rapide de l'arsenal protéique de l'embryon. L'accélération dans l'accroissement de la quantité de protéines extractibles résulte vraisemblablement de la mise en marche de la circulation sanguine. Ce phénomène modifie probablement l'équilibre enzymatique existant au sein de la masse vitelline endodermique et entraîne la digestion rapide de cette dernière.

Les synthèses protéiques ne se produisent pas de façon continue au cours du développement. Elles se répartissent au contraire en deux périodes distinctes de l'ontogenèse. La première période d'activité anabolique intense est simultanée aux mouvements morphogénétiques (stades 8–19). La seconde période se situe entre le stade 31 et la prise de nourriture. Les synthèses précoces produisent des protéines caractéristiques de chaque tissu. Il s'agit peut-être de protéines de structure servant à la différenciation des diverses ébauches.

D'après nos expériences, les protéines embryonnaires se répartissent dans la jeune gastrula suivant un gradient animal-végétatif. Le gradient observé intéresse à la fois la quantité et la nature des protéines distribuées dans les diverses parties de l'embryon.

Au cours de la gastrulation, le contenu protéique de l'ectoderme subit d'importantes modifications.

1. L'ectoderme animal compétent de la jeune gastrula acquiert vers le stade 8 deux bandes protéiques particulières. Celles-ci se réduisent progressivement à mesure que la compétence de l'ectoderme diminue.
2. Dès le stade 11, des différences apparaissent dans la constitution protéique de l'ectoderme ventral et de l'ectoderme neural. Ces différences se révèlent avant qu'il soit histologiquement possible de distinguer l'un de l'autre l'ectoderme ventral et le tissu nerveux présomptif.
3. L'ectoderme en voie de différenciation nerveuse présente une bande protéique de mobilité électrophorétique très faible. La présence de cet élément est peut-être en relation avec les phénomènes d'induction.

Selon toute vraisemblance, les protéines solubles qui apparaissent au cours de l'ontogenèse proviennent d'une manière plus ou moins directe du matériel vitellin. S'il en est ainsi, la courbe de la fig. 8 indique une modification de la vitesse de résorption du vitellus vers le stade 31. Ce phénomène peut être interprété comme une indication de l'existence de deux types de vitellus dans l'embryon. Le premier type, localisé dans les organes axiaux (ectoderme et mésoderme) serait seul utilisé dans la période de l'ontogenèse qui s'étend de la fécondation au stade 31. Après ce dernier stade, le second type de matériel de réserve

— le vitellus endodermique — commence à être digéré. Cette seconde espèce de matériel vitellin correspondrait au vitellus extra-embryonnaire des oiseaux. La pente légèrement décroissante des deux segments de la courbe n° 8 indique un ralentissement progressif de la vitesse de mise en solution de chaque type de vitellus. Cette perte de vitesse semble due à la diminution de la quantité de matériel disponible d'une part dans les organes axiaux et d'autre part dans l'endoderme.

Panijel (1950) a signalé l'existence dans l'œuf mûr de deux sortes de plaquettes vitellines. Ces deux espèces diffèrent par leur taille et par leurs propriétés chimiques. La première variété est formée de granules vitellins de petite dimension. Elle se rencontre dans la partie animale (futur ectoderme) et dans la zone intermédiaire (futur mésoderme) de l'œuf. La seconde variété comprend de grosses plaquettes localisées dans la région végétative (futur endoderme) de l'œuf et aussi dans la zone intermédiaire. La première partie (0–31) de la courbe n° 8 correspondrait à la digestion des petites plaquettes de Panijel. La seconde section du même diagramme serait une mesure de la vitesse d'utilisation des grosses plaquettes (beaucoup plus abondantes). Le premier type de matériel vitellin pourrait jouer un rôle important dans l'organogenèse primaire et en particulier dans les phénomènes d'invagination et d'induction.

RÉSUMÉ

1. Les protéines extraites d'embryons de *P. waltii* (stades 0–37) ont été soumises à l'électrophorèse sur acétate de cellulose. Plusieurs tissus embryonnaires ont été également étudiés par la même méthode.

2. Au cours du développement, le spectre électrophorétique d'embryons entiers acquiert une nouvelle bande au début de la gastrulation. Peu de changements interviennent dans le protéinogramme 'total' jusqu'au stade 31. Après ce stade, le spectre électrophorétique se diversifie rapidement.

3. La quantité totale de protéines extractibles par embryon s'accroît lentement du stade 0 au stade 31 et beaucoup plus rapidement à partir du stade 31. Cette accélération est probablement due à la mise en marche de la circulation sanguine.

4. Dès le début de la gastrulation, des différences apparaissent dans la constitution protéique des divers feuillets embryonnaires. Les protéines semblent se répartir dans la jeune gastrula selon un gradient dorso-ventral.

5. L'ectoderme compétent et l'ectoderme en voie de neurulation possèdent des bandes protéiques particulières.

6. Le contenu protéique de l'ectoderme ventral et celui de l'ectoderme neural se montrent différents dès la seconde partie de la gastrulation.

7. Chaque tissu embryonnaire (sauf l'endoderme) acquiert de nouvelles bandes protéiques au cours de la neurulation. Ces bandes sont différentes pour chaque tissu étudié.

8. L'embryon possède probablement deux types de vitellus, digérés non pas

en même temps mais en deux phases successives. Le premier type serait localisé dans les organes axiaux. Le second type, contenu dans l'endoderme, ne serait résorbé qu'à partir du stade 31.

SUMMARY

1. Saline extracts of embryos of stages 0–37 (*Pleurodeles waltii*) were subjected to electrophoresis on cellulose acetate. Various embryonic tissues were studied in the same way.

2. A new band appears in the electrophoretic pattern of whole embryos at the onset of gastrulation. Only minor changes can be detected until stage 31. Thereafter, the electrophoretic pattern becomes rapidly more complicated.

3. From stage 0 to stage 31, there is a slow increase of the overall amount of extractable protein per embryo. After stage 31, soluble proteins appear at an increased rate. This acceleration is probably due to the starting of blood circulation.

4. The various embryonic tissues already have a different protein constitution at the beginning of gastrulation. The proteins seem to be distributed in the early gastrula according to a dorso-ventral gradient.

5. Competent ectoderm and neurulating ectoderm contain particular protein bands.

6. From the second part of gastrulation onwards, the protein content of ventral ectoderm is different from that of neural ectoderm.

7. Every embryonic tissue (except endoderm) acquires new protein bands during neurulation. These bands are different in each tissue.

8. The embryo probably has two types of yolk. These two types are not digested at the same time but successively. The first type is probably located in the axial organs. The second type is contained in the endoderm and seems to be digested only after stage 31.

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Perturbations indirectes de l'organogénèse cardio-vasculaire obtenues par la destruction aux rayons X de territoires situés en arrière de l'ébauche du cœur

par NICOLE LE DOUARIN[1]

Laboratoire d'Embryologie expérimentale du Collège de France et du C.N.R.S., Paris. (Directeur: Professeur Ét. Wolff)

AVEC UNE PLANCHE

De nombreuses techniques permettent de perturber l'organogénèse du cœur et des arcs aortiques chez les embryons de Vertébrés. Les unes agissent sur l'ensemble de l'organisme embryonnaire: action de la thyroxine (Bauman & Pfister, 1936), carence vitaminique de la femelle gestante (Wilson & Warkany, 1949) ou hypoxie temporaire de l'œuf en incubation (Rubsaamen & Schellong, 1953). D'autres techniques, au contraire, ont été appliquées directement sur le système artériel. Elles ont consisté en des destructions par l'électrocautère (Bremer, 1928) ou en des ligatures (Stéphan, 1952). Elles ont eu pour conséquence non seulement des perturbations du système artériel, mais également des malformations cardiaques. Enfin, l'irradiation aux rayons X du cœur de l'embryon de Poulet, réalisée suivant la technique mise au point par Ancel & Ét. Wolff (1934), a permis d'obtenir des embryons présentant d'importantes anomalies du cœur et des vaisseaux (G. Le Douarin & N. Le Douarin, 1959; G. Le Douarin, 1960).

Des expériences de radiodestructions, chez l'embryon de Poulet, de larges bandes transversales intéressant les territoires situés en arrière de l'ébauche cardiaque produisent également des malformations importantes du système cardio-vasculaire, bien que le cœur ait été protégé. Cette méthode permet l'étude de l'influence de l'environnement sur l'organogénèse cardiaque.

TECHNIQUE

La technique utilisée a été décrite dans un travail antérieur (N. Le Douarin, 1960, 1961).

Seuls les embryons ayant survécu au moins jusqu'au 7ème jour de l'incubation ont été retenus pour cette étude et l'injection à l'encre de Chine du système

[1] *Author's address*: Laboratoire d'Embryologie expérimentale du Collège de France, 49bis, Avenue de la belle Gabrielle, Nogent-sur-Marne, Seine, France.

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cardio-vasculaire a été pratiquée pour favoriser l'observation du système artériel. Les cœurs ont été étudiés à l'aide de coupes transversales pratiquées à différents niveaux.

RÉSULTATS

Deux séries expérimentales ont été réalisées.

Irradiation de l'embryon en arrière d'un somite déterminé

Dans ces expériences, la partie toute postérieure de l'embryon a été protégée, pour que l'allantoïde puisse se développer et permettre ainsi une survie dépassant 6 jours d'incubation. La morphologie des animaux obtenus par cette méthode a été décrite dans un travail antérieur (N. Le Douarin, 1960). Les deux parties protégées se développent en s'unissant l'une à l'autre par un court pédicule.

Le tableau 1 indique les interventions pratiquées.

TABLEAU 1

Niveau de l'irradiation. En arrière du . . .	*Stade (Nombre de somites)*	*Nombre de cas*	*Survie ⩾ 7 jours*
2ème somite	9 à 12	100	14
3ème somite	10 à 15	30	5
4ème somite	15 à 19	30	8
6ème somite	18 à 22	15	3
15ème somite	18 à 22	40	10

Anomalies du système artériel

La proportion d'embryons présentant des arcs aortiques anormaux diminue lorsque la limite antérieure de l'irradiation s'éloigne du cœur, comme le montre le tableau 2.

TABLEAU 2

Niveau de l'irradiation. En arrière du . . .	*Arcs normaux*	*Arcs anormaux*
2ème somite	0	14
3ème somite	0	5
4ème somite	1	7
6ème somite	2	1
15ème somite	10	0

On voit qu'une irradiation en arrière du 15ème somite, donc respectant le territoire embryonnaire destiné à donner le cou, ne perturbe pas l'évolution normale des arcs aortiques.

Les anomalies vasculaires observées réalisent un dispositif presque constant (fig. 2), s'éloignant considérablement de la disposition normale schématisée sur la fig. 1. Les arcs 3 sont réduits, parfois même ils ne sont pas fonctionnels et ne

subsistent qu'à l'état de reliquats fibreux. Les artères carotides communes sont formées par l'arc 4 à droite et l'arc 6 à gauche. L'aorte dorsale est formée par l'arc 6 droit seul.

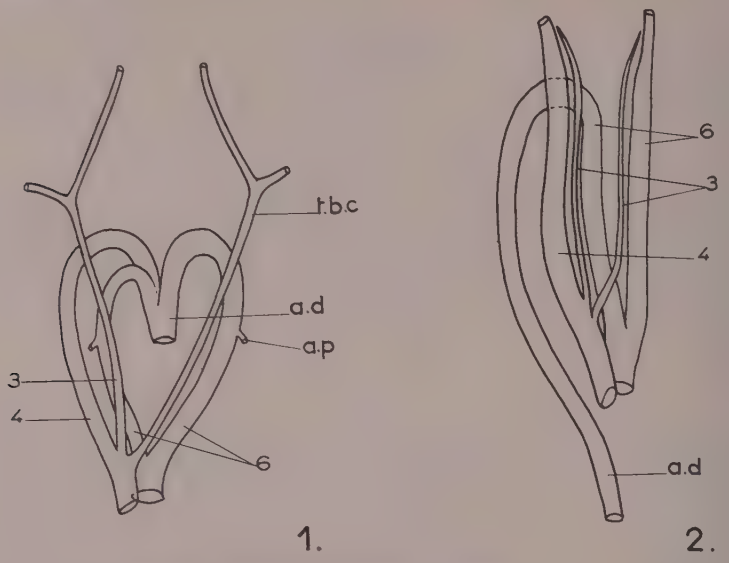

FIG. 1. Arcs aortiques d'un embryon normal de 7 jours.
FIG. 2. Arcs aortiques d'un embryon de 7 jours, ayant subi la radiodestruction d'un territoire étendu situé en arrière du cœur (1ère série expérimentale). Les arcs 3 sont réduits et les artères carotides sont formées par l'arc 4 à droite et l'arc 6 à gauche. Les membres antérieurs sont détruits par l'irradiation; il n'y a pas d'artères sous-clavières. La crosse aortique est formée par l'arc 6 droit.
3,4,6: 3ème, 4ème, et 6ème arcs aortiques; *a.d*, aorte dorsale; *a.p*, artère pulmonaire; *t.b.c*, tronc brachiocéphalique.

Malformations cardiaques

Elles intéressent la morphologie externe et la constitution interne de l'organe. Le tableau 3 indique leur fréquence.

TABLEAU 3

Niveau de l'irradiation. En arrière du . . .	*Cœurs normaux*	*Cœurs anormaux*
2ème somite	0	14
3ème somite	1	4
4ème somite	2	6
6ème somite	2	1
15ème somite	10	0

La fréquence des malformations décroît lorsque le niveau antérieur de l'irradiation s'éloigne du cœur. L'irradiation en arrière du 15ème somite est sans effet sur l'organogénèse cardiaque.

Anomalies de la morphologie externe

1. *Spiralisation du tube cardiaque.* Normalement, les mouvements morphogénétiques du cœur amènent le bulbe artériel sur la ligne médio-ventrale. Les anomalies observées consistent en une absence complète de rotation du bulbe artériel (fig. 3) (17 cas), ou en un inachèvement de cette rotation (fig. 4) (6 cas).

On observe alors un développement incomplet de l'oreillette droite; en effet le cloisonnement de l'atrium n'a pas été suivi, à droite, du développement de l'auricule faisant normalement déborder l'oreillette sur le ventricule. La présence du bulbe artériel à droite de l'atrium semble présenter une gêne mécanique empêchant ce développement.

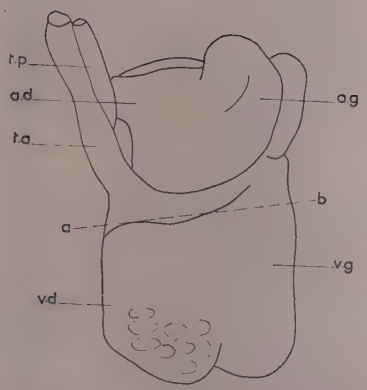

FIG. 3

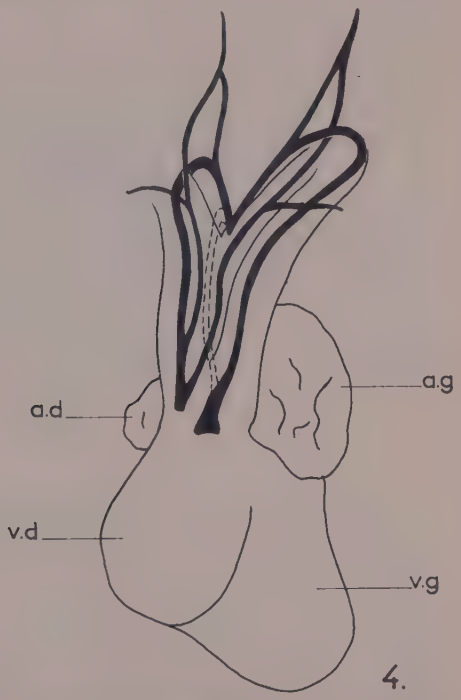

FIG. 4

FIG. 3. Cœur d'un embryon de 9 jours montrant un bulbe artériel resté à droite de l'atrium (*a–b* niveau de la coupe représentée en la fig. 7).

FIG. 4. Cœur et départ des gros vaisseaux d'un embryon de 9 jours montrant un déplacement inachevé du bulbe artériel vers la ligne médio-ventrale. L'atrium droit est réduit. Le ventricule droit est en saillie sur le ventricule gauche.

a.d, atrium droit; *a.g*, atrium gauche; *t.a*, tronc aortique; *t.p*, tronc pulmonaire; *v.d*, ventricule droit; *v.g*, ventricule gauche.

Or au cours de l'organogénèse normale du cœur, l'oreillette gauche présente jusqu'au 5ème jour de l'incubation une taille supérieure à celle de l'oreillette droite. A la fin du 4ème jour le bulbe artériel atteint la ligne médio-ventrale, et dès ce moment la croissance de l'oreillette droite devenant plus active que celle de

l'oreillette gauche, elle rattrape et dépasse même, au cours du 5ème jour, la taille de cette dernière. Il semble que la disposition anormale réalisée dans ces expériences apporte un argument en faveur de l'hypothèse selon laquelle le retard temporaire du développement de l'oreillette droite est dû à l'obstacle représenté par le bulbe artériel situé du côté droit au début de l'organogénèse cardiaque.

2. *Aspect des ventricules.* Leur surface externe peut être irrégulière et bosselée (Planche, fig. B). Ils peuvent être séparés par un sillon profond. Enfin, le ventricule droit peut faire saillie ventralement sur le gauche (fig. 4).

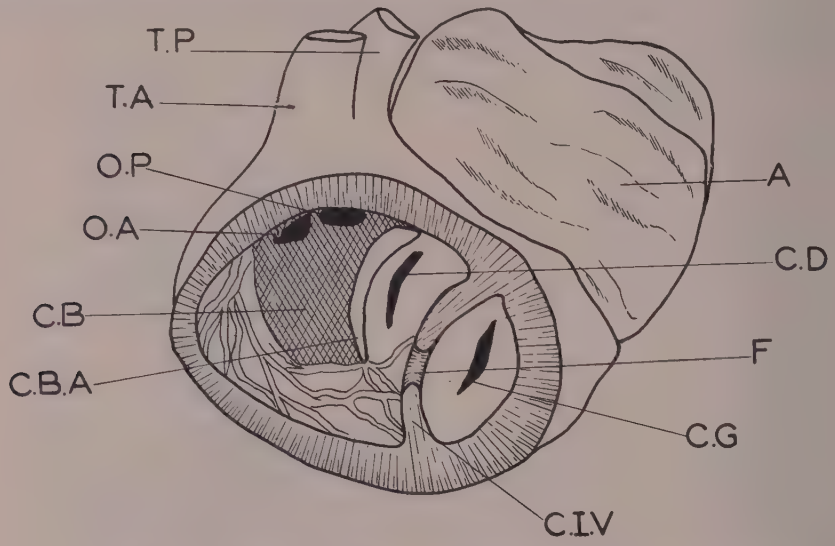

FIG. 5. Schéma de la constitution interne du cœur représenté fig. 3 (coupé transversalement au niveau *a–b*). Vue vers les oreillettes. A, atrium; C.B, cavité bulbaire; C.B.A, crête bulbo-atriale; C.D, C.G, canaux atrio-ventriculaires droit et gauche; C.I.V, cloison inter-ventriculaire; F, foramen inter-ventriculaire; O.A, O.P, orifices aortique et pulmonaire; T.A, T.P, troncs aortique et pulmonaire.

3. *Le péricarde.* Lorsque l'irradiation produit une cœlosomie, le péricarde est fréquemment soudé au bord antérieur de la paroi du corps, ce qui donne au cœur une position inversée: sa face dorsale devenant ventrale et sa pointe normalement caudale devenant crâniale (Planche, fig. A).

Anomalies de la constitution interne

Les malformations les plus importantes s'observent lorsque le bulbe artériel est resté à droite de l'atrium. La constitution interne du cœur est alors la suivante:

Le ventricule droit montre un volume supérieur à celui du ventricule gauche et se prolonge par un bulbe artériel dont la cavité interne est vaste, indivise et à parois lisses; les crêtes qui sont à l'origine de son cloisonnement ne sont même pas amorcées. La cloison inter-ventriculaire reste incomplète, permettant ainsi au ventricule gauche de chasser son sang dans le bulbe par la voie du foramen inter-ventriculaire qui n'est pas obturé. La cloison aortico-pulmonaire est totalement

absente au niveau du bulbe, seul le tronc artériel est divisé en deux par une cloison légèrement spirale qui amène le tronc aortique ventralement par rapport au tronc pulmonaire.

La crête bulbo-atriale n'a pas subi sa régression normale et sépare ainsi le bulbe artériel de l'orifice atrio-ventriculaire droit (fig. 5).

La torsion du tube cardiaque s'arrête au stade où le cœur réalise une forme en U, c'est-à-dire celle de l'organe au 3ème jour de l'incubation. Le cœur a augmenté de taille tout en conservant cette disposition.

Au cours de l'organogénèse normale, le cœur se déplace vers l'arrière par suite de la flexure de la tête et de l'allongement du cou. La radiodestruction des somites destinés à former le cou l'amène donc à évoluer dans un territoire extrêmement perturbé; il en résulte que ses mouvements morphogénétiques ne s'accomplissent pas normalement et les anomalies internes constatées sont les conséquences de l'inachèvement de ces mouvements. Il apparaît donc que certaines parties du cloisonnement cardiaque sont tributaires d'un déroulement normal des mouvements morphogénétiques. Il s'agit de la cloison aortico-pulmonaire bulbaire et de la formation de la base de la rampe aortique, c'est-à-dire de la transformation en canal du foramen inter-ventriculaire initial par le matériel endocardique du coussinet endocardique médian atrio-ventriculaire. Par contre, en dépit de l'inachèvement de la torsion du tube cardiaque et des courant sanguins anormaux qu'il entraîne, certains processus morphogénétiques peuvent s'accomplir: le cloisonnement de l'atrium, celui du ventricule primitif en cavités droite et gauche, la séparation du tronc artériel en deux vaisseaux à parois musculaires indépendantes.

Irradiation d'une bande étroite située en arrière du cœur

Les expériences réalisées sont résumées sur le tableau 4.

TABLEAU 4

Niveau de l'irradiation		*Stade (Nombre de somites)*	*Nombre de cas*	*Survie ⩾ 7 jours*
Limite antérieure. En arrière du . . .	*Limite postérieure. En arrière du . . .*			
2ème somite	10ème somite	10 à 12	30	13
2ème somite	6ème somite	10 à 12	10	8
4ème somite	15ème somite	15 à 19	30	10
6ème somite	15ème somite	15 à 20	30	11

Anomalies des arcs aortiques

Leur fréquence dépend du niveau antérieur de l'irradiation et également de l'étendue du territoire détruit.

Les arcs aortiques sont anormaux dans 26 cas sur 42. La plus grande fréquence correspond aux irradiations antérieures: entre les 2ème et 10ème somites d'une part et les 4ème et 15ème somites d'autre part. L'irradiation d'une bande étroite, limitée à 4 somites, même située juste en arrière du cœur, provoque une proportion assez réduite de malformations vasculaires.

TABLEAU 5

Niveau de l'irradiation			
Limite antérieure. En arrière du . . .	*Limite postérieure. En arrière du . . .*	*Arcs normaux*	*Arcs anormaux*
2ème somite	10ème somite	2	11
2ème somite	6ème somite	5	3
4ème somite	15ème somite	2	8
6ème somite	15ème somite	7	4

Les systèmes vasculaires anormaux obtenus dans cette série expérimentale présentent une disposition presque constante (fig. 6). Les arcs 3 sont d'importance réduite. Ils s'unissent aux racines aortiques (issues de l'arc 4 à droite et de l'arc 6 à gauche) pour former les artères carotides communes. L'arc 4 droit conserve par ailleurs sa destinée normale: il forme la crosse aortique.

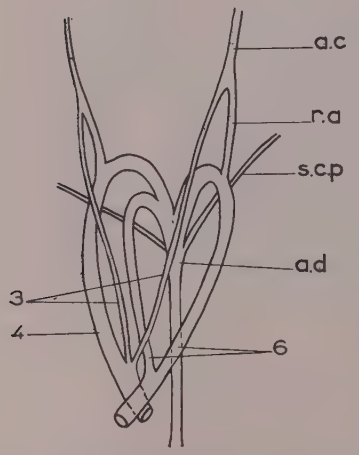

FIG. 6. Arcs aortiques d'un embryon de 7 jours ayant subi la radiodestruction d'une bande transversale étroite située en arrière du cœur (2ème série expérimentale). Les arcs 3 sont réduits et unis aux racines aortiques persistantes pour former les artères carotides, les artères sous-clavières sont les vaisseaux primaires. La crosse aortique est normalement formée par l'arc 4 droit. 3, 4, 6 = 3ème, 4ème et 6ème arcs aortiques; *a.c*, artère carotide commune; *a.d*, aorte dorsale; *r.a*, racine aortique; *s.c.p*, artère sous-clavière primaire.

En conclusion, les anomalies vasculaires observées sont moins importantes que celles obtenues dans la première série expérimentale. Mais elles intéressent également surtout les troncs brachio-céphaliques; les arcs 3 ne fournissent pas la totalité de l'irrigation de la partie du corps située en avant du cœur (tête, cou et membres antérieurs), la persistance des racines aortiques pallie à cette insuffisance.

Malformations cardiaques

Elles ont été observées dans 21 cas sur 42; les proportions obtenues pour les différents territoires irradiés ne présentent pas de différences significatives.

Les anomalies sont le plus souvent moins importantes que celles obtenues dans la première série expérimentale. Ainsi par exemple, le bulbe artériel ne reste à droite de l'atrium que dans 3 cas sur 42 animaux étudiés, dans 9 cas il amorce la rotation qui l'amène normalement en position médio-ventrale; mais le plus souvent (30 cas sur 42) il achève cette rotation.

Du point de vue de la constitution interne les malformations rencontrées le plus fréquemment sont les suivantes:

La cloison aortico-pulmonaire du bulbe laisse subsister une communication

entre les rampes artérielles sous la forme d'un orifice plus ou moins important.

Le foramen inter-ventriculaire n'est pas obturé (11 cas).

La figure 7 représente un cœur où ces deux anomalies coexistent.

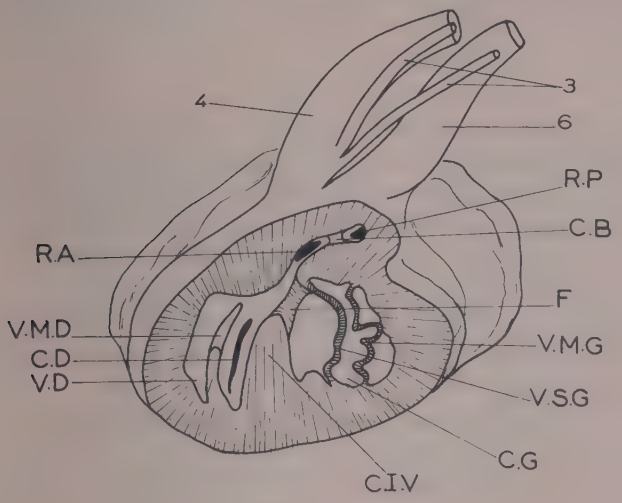

FIG. 7. Schéma de la constitution interne du cœur d'un embryon de 10 jours de la 2ème série expérimentale (irradiation d'une bande étroite située en arrière du cœur). Vue vers les oreillettes. La cloison bulbaire est incomplète, les rampes aortique et pulmonaire communiquent. Persistance du foramen inter-ventriculaire. 3, 4, 6, 3ème, 4ème et 6ème arcs aortiques; C.B, crête bulbaire; C.D, C.G, canaux atrio-ventriculaires droit et gauche; C.I.V, cloison inter-ventriculaire; R.A, R.P, rampes aortique et pulmonaire; V.D., ventricule droit; V.M.D, V.M.G, valvule marginale droite et gauche; V.S.G, valvule septale gauche.

CONCLUSION

La destruction par les rayons X de territoires situés en arrière de l'ébauche cardiaque a été réalisée chez des embryons de Poulet aux stades de 9 à 22 somites.

Ces expériences produisent des anomalies du cœur et des arcs artériels, qui ont été étudiées chez les embryons ayant dépassé le 7ème jour de l'incubation.

Les malformations sont d'autant plus fréquentes et importantes que le niveau antérieur de l'irradiation se situe plus près du cœur et que le territoire détruit est plus étendu.

Les anomalies vasculaires intéressent surtout les troncs brachio-céphaliques et, moins fréquemment, la crosse de l'aorte.

Au niveau du cœur les modifications apportées au territoire où évolue l'ébauche de cet organe provoquent des perturbations des mouvements morphogénétiques, en particulier de la rotation du bulbe artériel. Certaines parties

du cloisonnement cardiaque s'en trouvent affectées : la cloison aortico-pulmonaire ne se forme pas ou reste imparfaite, le foramen interventriculaire ne s'obture pas. Le cœurc onserve donc à certains égards l'organisation qui est la sienne à un stade précoce du développement. Par contre, la cloison inter-auriculaire et la cloison inter-ventriculaire évoluent normalement et semblent donc ne pas dépendre de l'accomplissement normal des mouvements morphogénétiques du cœur.

SUMMARY

Experimental destruction by X-radiation of wide transverse strips situated behind the heart rudiment has been produced in chick embryos at 9- to 22-somite stages. Cardio-vascular malformations resulted. Embryos surviving at least to the 7th day of incubation were studied. The arterial system was injected with India ink and the heart cut transversely at different levels.

The results were as follows:

1. Malformations were more frequent and serious the closer the anterior margin of the irradiated strip approached the heart and the more extensive the destroyed region was.

2. Arterial anomalies affected mainly the brachiocephalic trunks, and, less frequently, the arch of the aorta.

3. Disturbances of morphogenetic movements, especially of the rotation of the arterial bulbus, occurred in the development of the heart itself. The partitioning of the heart was in places affected: the pulmo-aortic septum of the bulb failed to form or remained incomplete, and the interventricular foramen did not close. The heart, therefore, in some respects maintained the organization which it had at an earlier stage. In contrast, the interatrial and interventricular septa developed normally and seemed, therefore, not to depend on the normal completion of the morphogenetic movements of the heart.

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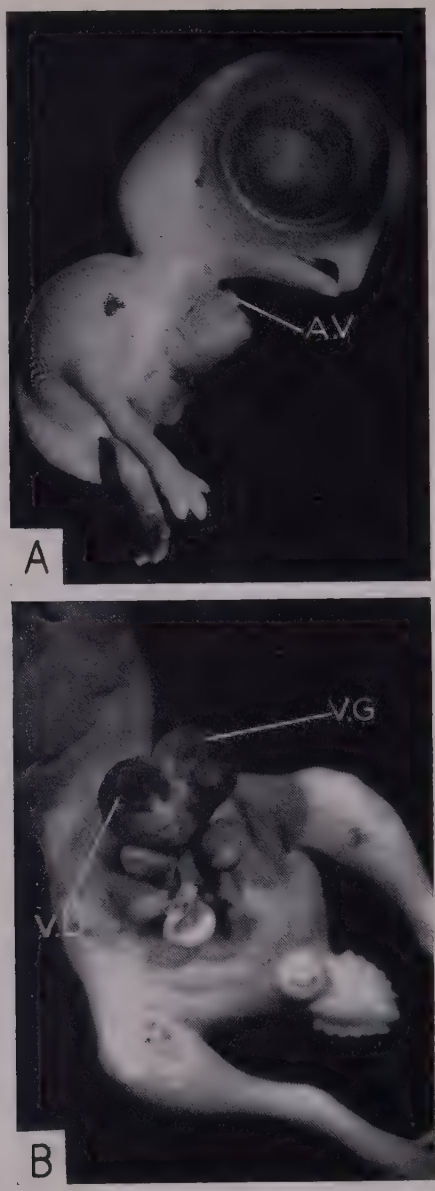
A.V
A
V.G
V.
B

EXPLICATION DE LA PLANCHE

FIG. A. Vue d'un embryon de la première série expérimentale. Cœlosomie. Le cœur présente une surface bosselée et une situation inversée par suite de la soudure du péricarde ventral à la limite antérieure de la cœlosomie. A.V, apex ventriculaire. FIG. B. Vue ventrale d'un embryon de la 1ère série expérimentale. Cœlosomie. Les deux ventricules sont séparés par un sillon profond. V D, ventricule droit; V.G, ventricule gauche.

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Atypical Spindles in Reinnervated Rat Muscles

by P. HNÍK *and* J. ZELENÁ[1]

From the Institute of Physiology, Czechoslovak Academy of Sciences, Prague

WITH PLATE

In young rats, following lesions of a peripheral nerve at birth, immature muscle spindles disintegrate during a short transition period (Zelená, 1957), and even after several months of reinnervation the reinnervated muscles are usually found to contain no spindles (Zelená & Hník, 1960). Nevertheless, occasional small spindles of atypical structure are observed in some of these muscles. In the present report, the structure and morphological characteristics of these atypical spindles were studied and compared with normal spindles. Since differences in fast and slow muscles may include differences in muscle receptors (Voss, 1937; Maruseva, 1947; Hagbarth & Wohlfahrt, 1952; Freimann, 1953; Cooper, 1960), the number, size, and distribution of spindles were studied in two muscles representing the two functional groups.

The origin of atypical spindles in the reinnervated muscles is not clear. These spindles could either differentiate anew under the influence of regenerating sensory nerve fibres, or they could originate from spindles reinnervated before their ultimate disintegration had taken place. Since atypical spindles are so very rare compared with the large number of regenerated afferent nerve fibres present in the muscle, the former explanation is considered to be less probable (Zelená & Hník, 1960). In order to investigate the latter possibility, a quantitative study was therefore made of muscles 10 days after nerve section at birth, to find out whether they contain spindles which could be reinnervated afterwards.

METHODS

I. In one group of animals the sciatic nerve was crushed unilaterally with narrow forceps in new-born rats. Five months after reinnervation both the reinnervated and control soleus and extensor digitorum longus were excised, weighed, and fixed in 10 per cent. formol. After embedding in paraffin, complete series of transverse sections from reinnervated muscle and complete or inter-

[1] *Authors' address*: Československá Akademie Věd, Fysiologický Ústav, Praha-Dejvice, Na Cvičišti č. 2, Czechoslovakia.

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rupted series of normal muscles were made, the thickness of sections being either 10 or 20 μ. The sections were stained with haematoxylin-eosin, or according to Van Gieson.

All normal spindles of control muscles and atypical spindles found in reinnervated muscles were characterized by the following parameters: length, maximal diameter of the spindle including the capsule, number and diameter of intrafusal fibres, and structure of the equatorial zone when present. The above dimensions were measured directly with the aid of an ocular micrometer at a magnification of $\times 500$, with the exception of length, which was calculated from the number and thickness of sections. Some difficulty was encountered in determining the total length of the spindle and it was therefore necessary to delimit the longitudinal dimensions by arbitrary reference points. The length was measured from two extreme sections in which the capsule and beginning of the perifascicular space were just discernible. Since considerable shrinkage due to fixation and paraffin embedding could be expected, an attempt was made to ascertain the difference between values measured and actual dimensions *in vivo*. After measuring the length of muscles before and after fixation and embedding, a shortening of 10–15 per cent. was usually found. In two solei and two extensor digitorum longus muscles reconstruction was made of the longitudinal distribution of spindles and of their localization in cross-section.

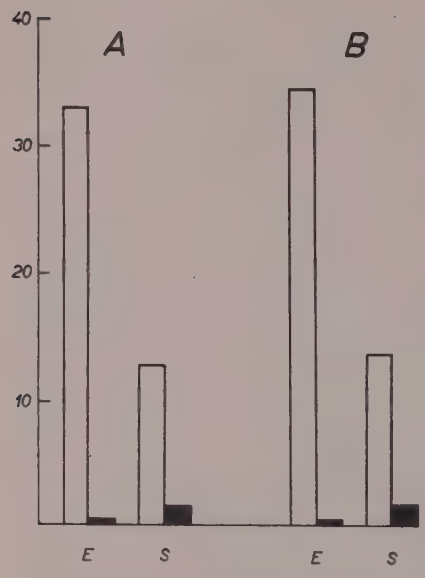
II. In the second group of animals the sciatic nerve was sectioned unilaterally in new-born rats (Zelená, 1957). Ten days later (i.e. at the time when reinnervation usually takes place following crushing of the nerve) the soleus and extensor digitorum longus were excised, fixed in formol, and embedded in paraffin. The number of spindles in normal muscles and of spindle remnants in the denervated muscles was counted in complete series of sections stained with haematoxylin-eosin or according to Van Gieson.

RESULTS

The number and structure of spindles in the normal extensor digitorum longus and soleus muscles

In the two muscles investigated, considerable differences were encountered in the number of spindles. The average number in the soleus and extensor digitorum longus muscles was $12{\cdot}7 \pm 0{\cdot}95$ (6 muscles) and $32{\cdot}9 \pm 1{\cdot}55$ (7 muscles) respectively (Text-fig. 1A). Thus in the soleus there is one spindle to 7·18 mg. wet muscle weight, while in the extensor digitorum longus there is one spindle to 3·75 mg. This means that in the former 0·1 g. of muscle tissue contains 13·1 spindles and in the latter 24·8 spindles. There is also a difference in the spatial distribution of spindles in these two muscles. In the soleus the spindles are fairly uniformly distributed throughout the muscle while in the extensor digitorum longus spindles were to be found exclusively in the anterior half (Text-fig. 2). Both muscle regions adjoining the tendon are devoid of muscles spindles as in human

muscles (Voss, 1956*a*). In a few cases two consecutive spindles were in close contact so that their capsules were fused together side-to-side at the ends (Plate, fig. 5); fascicles of intrafusal fibres were never found to be interconnected.



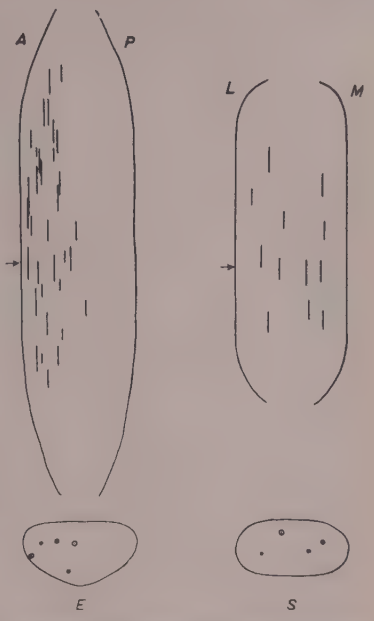
TEXT-FIG. 1. A, the number of spindles in normal (white columns) and reinnervated muscles (black columns) of the rat 5 months after crushing the sciatic nerve at birth. B, the number of spindles in normal (white columns) and denervated muscles (black columns) of the rat 10 days after neurotomy at birth. E, m. extensor digitorum longus; S, m. soleus. Ordinate: average number of spindles.

The average length of spindles was found to be 1·1 mm. (for further details see Table 1), but the range between minimum and maximum values was wide, being 280–2,320 μ in the extensor digitorum longus and 700–1,740 μ in the soleus. From the distribution curves in Text-fig. 3 it is evident that the lengths of spindles in the soleus are more uniform than in the extensor digitorum longus and that the soleus does not contain dwarf spindles of the smallest category (200–600 μ in length).

The spindle diameter including the capsule was measured at the equatorial zone. Part of the spindles at this level are either round or oval in shape, the intrafusal fibre bundle being placed excentrically, with most of the supporting connective tissue strands broken. Some spindles, however, have an irregular shape due to the infoldings of the capsule which is probably, in most cases, a fixation artefact caused by the retraction of connective tissue strands. The maximum diameters of spindles in the equatorial zone may thus be subject to some error, and this may explain differences in the mean values found between individual muscles (see Table 1). However, when the mean diameter of all spindles measured is calculated, these differences are no longer apparent and the standard error of the mean is relatively small (50·9$\pm$2·24 μ and 55·2$\pm$2·02 μ in the soleus and extensor digitorum longus, respectively).

The number of intrafusal muscle fibres was relatively constant in the soleus muscle, its spindles consisting of four, sometimes three, intrafusal fibres, the average number being 3·8 fibres per spindle. In the extensor digitorum longus the average number of fibres was very similar (3·7 fibres per spindle), although occasional small spindles (1–3 in a muscle) containing 1–2 intrafusal fibres could be found in this muscle. Three to four intrafusal fibres per spindle thus seem to be species specific for the rat leg-muscles. The diameter of intrafusal fibres in the region of the equatorial zone forming the nuclear bag is 10·9 and

10·5 μ in the soleus and extensor digitorum longus respectively. The nuclear bag, which is 80–100 μ long, is usually formed simultaneously in two, rarely in three, fibres present in a spindle. The remaining one or two fibres may exceptionally form a nuclear bag at an adjacent level. In most spindles the structure of these fibres remains myotubal and the nuclear bag is absent. Their diameter is then much smaller, the mean values being 6·5 and 6·0 μ respectively.



TEXT-FIG. 2. Diagram of the distribution and length of spindles in the normal left extensor digitorum longus (*E*) and the soleus (*S*), as reconstructed from serial sections. *A–P* anterior and posterior surface; *L–M*, lateral and medial surface. Arrows indicate the level of cross-section given below.

Structure and dimensions of atypical spindles in reinnervated muscles

Atypical spindles (Plate, figs. 6, 8, 9) found after a reinnervation period of 5 months are extremely rare in the extensor digitorum longus, the average number being 0·55±0·55 (9 muscles); the mean value of the soleus muscle was a little higher, being 1·50±0·22 spindles per muscle (6 muscles). These spindles are localized near the nerve entrance and they differ at first sight from normal spindles by being greatly undersized. Their length is approximately one-third of that of normal spindles and the maximum diameter across the equatorial zone is roughly one-half in the extensor digitorum longus and three-quarters in the soleus (Table 2).

In contrast to normal spindles, which contain an average of 3·7–3·8 intrafusal muscle fibres, the number of intrafusal fibres is reduced to 2 in the extensor digitorum longus and to 1·6 in the soleus. Besides these quantitative differences, atypical spindles are usually incompletely differentiated. The nuclear bag—the most conspicuous spindle structure—is not present in its typical form, but it is represented either by a continuous or interrupted chain of nuclei arranged in the centre of the fibre in single file. In 2 spindles out of 8 thus investigated fibres were extrafusal in character, i.e. their nuclei were localized on the circumference of the fibre and their diameter was almost double that of intrafusal fibres in the other atypical spindles (Plate, figs. 8, 9). In spite of this, however, the maximum diameter was practically the same as in the latter. The structure and shape of the connective tissue capsule and supporting strands in the equatorial zone did not differ from those of the normal spindles.

As has already been pointed out, the occurrence of atypical spindles in

reinnervated muscles is very rare. Because of this, the question of the mechanism of their origin has arisen.

TABLE 1

Parameters of normal spindles in control muscles

	M. soleus		*M. extensor digitorum longus*	
	1	2	1	2
	Mean values		*Mean values*	
Weight of muscles in mg.	87	98	140	116
	92·5		128	
Number of spindles	15	12	34	35
	13·5		34·5	
Length of spindles	1,139±63·7	1,208±80·6	1,099±67·6	1,190±62
	1,177±52·2		1,144±45·8	
Diameter of spindles	53·4±2·94	49·1±3·16	46·5±2·22	64·1±2·66
	50·9±2·24		55·2±2·02	
Length of the equatorial zone	103±9·5	95±3·9	94·1±3·2	86·7±4·2
	97·6±4·12		90·9±2·51	
Number of intrafusal fibres	3·92±0·09	3·7±0·09	3·7±0·08	3·7±0·13
	3·8±0·08		3·7±0·06	
Diameter of fibres with nuclear bag	11·0±0·36	10·6±0·07	9·9±0·15	11·0±0·22
	10·9±0·24		10·51±0·14	
Diameter of fibres without nuclear bag	6·7±0·22	6·0±0·28	6·00±0·34	6·1±0·56
	6·5±0·14		5·98±0·45	

Numerals above vertical columns designate individual muscles investigated. Mean values were calculated from the two muscles ± standard error of the mean.

The number of spindle remnants in muscles 10 days after nerve section at birth

In order to obtain data concerning the possible explanation that atypical spindles originated from reinnervated spindle remnants not fully disintegrated at the beginning of reinnervation, the occurrence of these remnants was investigated 10 days after nerve section at birth. As spindle remnants were considered those structures in which the capsule containing intrafusal fibres could be discerned in several consecutive sections in the atrophic muscle tissue. Only in one case in the soleus muscle a relatively well-preserved spindle was found with a complete spindle-shaped capsule and intrafusal fibres surrounded by a distinct perifascicular space. The number of spindle remnants was 0·4 in the denervated extensor digitorum longus, whereas the control muscle contained an average of 34·4 spindles. In the soleus muscle an average of 1·6 spindle remnants were found, the number of spindles in normal muscles being 13·5 (Table 3 and Text-fig. 1B). These data correspond well with the number of

atypical and normal spindles found in these muscles after 5 months' reinnervation period.

TABLE 2

Parameters of atypical spindles in reinnervated muscles 5 months after crushing the nerve at birth

	M. soleus						*M. extensor digitorum longus*	
	1	2	3	3*a*	4	4*a*	1	2
Weight of muscles in mg.	24	33	22	—	—	—	36	37
Number of spindles .	1	1	2	—	2	—	1	1
Length . . .	480	500	560	incomplete	500	400	360	440
Diameter . .	40	42	33	41	34	40	27	29
Length of the equatorial zone	0	40	20	0	0	0	0	0
Number of intrafusal fibres	1	2	3	1	1	2	2	2
Diameter of fibres imitating nuclear bag	—	8	8	—	—	—	—	8
Diameter of fibres without nuclear bag	14	6	8 6	14	7	8 7	8 6	6

Numerals above vertical columns designate spindles in individual muscles (3*a* and 4*a*—second spindle in the same muscle).

TABLE 3

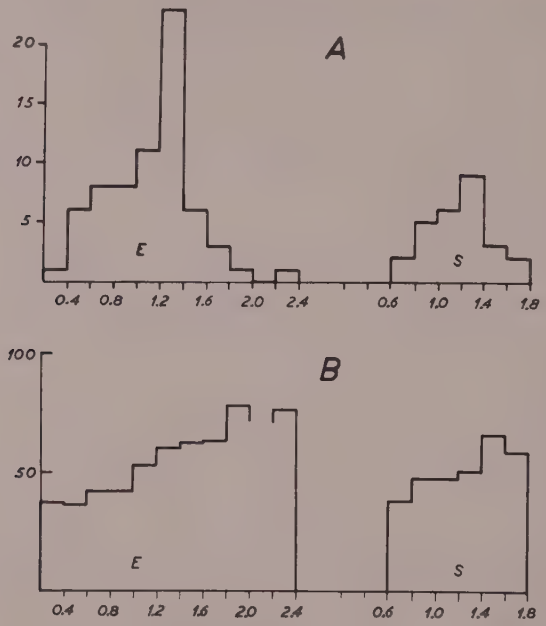
Number of spindles in muscles 10 days after nerve section at birth

	M. soleus		*M. extensor digitorum longus*	
	Denervated	*Control*	*Denervated*	*Control*
1 .	2	—	0	34
2 .	0	13	0	31
3 .	2	14	0	37
4 .	2	14	1	31
5 .	2	13	1	31
Mean values ±S.E.	1·6±0·4	13·5±0·28	0·40±0·25	34·4±1·6

DISCUSSION

A great difference in the average number of spindles was found in the normal rat muscles belonging to the flexor and extensor groups. The number of spindles in the extensor digitorum longus is more than twice as high as in

the soleus muscle, and the same ratio is found when relative values are compared, the number of spindles per decigramme wet muscle weight being 24·8 in the former and 13·1 in the latter.



TEXT-FIG. 3. A, the distribution curve of muscle spindles in two extensor digitorum longus (*E*) and soleus (*S*) muscles arranged in groups according to their length. Abscissa: length of spindles in mm. Ordinate: number of spindles in each group. B, diagram of spindle diameters in the corresponding groups. Abscissa: length of spindles in mm. Ordinate: diameter of equatorial zone.

Differences were also found in the distribution of spindles in the two muscles studied. Spindles in the soleus muscle are fairly uniformly distributed, with the exception of regions near the origin and tendon, where there are no spindles whatever. These regions are also without spindles in the extensor digitorum longus and this is evidently the rule in most muscles (Voss, 1956*a*). In contrast to the uniform distribution in the soleus, all the spindles in the extensor digitorum longus are concentrated in the anterior half of the muscle only. The significance of this finding is not evident. According to Gregor (1904) the accumulation of spindles in certain parts of the muscle in human foeti is due to local differences in the nerve supply. This conclusion was further corroborated by the recent findings of Barker & Chin (1960), who noted a close relationship between intramuscular nerve branches and the distribution of spindles in cat muscles.

The functional significance of these topographical differences in spindle distribution and intramuscular nerve supply is, however, not clear. As has been pointed out by Voss (1956*a*), the number of spindles contained in different muscles, or in different parts of the same muscle, is probably related to the amount of stretch to which the muscle, or its particular portion, is subjected (Freimann, 1953; Schulze, 1955; Voss, 1956*b*).

The absolute number of spindles in muscles of the rat is lower than in the cat or in man. On the other hand, the relative number per unit weight is higher by one order of magnitude than in the cat. In the cat, the absolute number found by Hagbarth & Wohlfahrt (1952) in the soleus was 56, and in the anterior tibial 57 spindles, according to Barker (1959), 70·7 spindles. However, the number of spindles per gramme, as calculated by Cooper (1960), is much higher in the slow soleus muscle than in the fast tibialis anterior (22 per gramme in the former and 13 per gramme in the latter), and this is considered to be a characteristic difference between the two functionally different groups. But this does not hold true in all cases, as was shown above for the rat extensor digitorum longus, which, although belonging to the fast muscle group, contained twice as many spindles, even when calculated per unit weight, as compared with the slow soleus muscle. It is thus evident that it is not possible to draw definite conclusions, unless the two functional groups are investigated as a whole in both species.

No significant differences were found in the average length of spindles in the soleus and extensor digitorum longus, although in other species (cat and frog) long spindles were described in slow tonic muscles and short spindles in muscles more phasic in function (Voss, 1937; Hagbarth & Wohlfahrt, 1952; Freimann, 1954; Cooper, 1960). However, the extensor digitorum longus contained a few short spindles (200–600 μ long) of correspondingly small diameter (Text-fig. 3), while the lengths of spindles in the soleus were more uniform and none of these dwarf spindles were to be found in this muscle.

Further, no difference was found in the mean diameter of the equatorial zone in the two muscles. When the 10–15 per cent. shrinkage caused by fixation and embedding is taken into account, the values obtained in our experiments (50·9 μ and 55·2 μ in the soleus and extensor digitorum longus, respectively) are comparable with the mean diameter of unfixed spindles (66·7 μ) isolated from frog muscles, as measured by Jahn (1959). According to this author, there is a direct correlation between the diameter of a spindle and the number of its intrafusal fibres. A similar relationship was observed by us in rat muscles, but the number of spindles containing less intrafusal fibres than four is relatively very small.

Granit & Homma (1959) classified spindles in rabbit muscles into three main groups—slow, medium, and fast. Slow muscles were found to contain slow and medium spindles, while fast muscles contained a large number of spindles of the fast category. However, this classification is arbitrary, as pointed out by the

authors themselves, since there is a gradual transition between the three groups. Although it is possible to draw only a loose analogy with the present experiments because of probable species differences, it is nevertheless worth noting that the slow soleus muscle contains a more homogenous group of spindles than extensor digitorum longus as far as their length, diameter, and number of intrafusal fibres is concerned (see Text-fig. 2). There is always the possibility that further differences might be found if spindle structure, especially in relation to innervation, were studied in more detail.

Atypical spindles found in reinnervated muscles exhibit not only dimensional but also structural differences as compared with normal spindles. These spindles resemble immature spindles of young animals with the interrupted or continuous chain of nuclei instead of a fully developed nuclear bag. Sometimes their intrafusal fibres do not contain any accumulation of nuclei, their structure being similar to that of extrafusal fibres and their diameter (14 μ) exceeding that of normal intrafusal fibres. These findings suggest that atypical spindles most probably originate from spindle remnants reinnervated before their disintegration had taken place during the denervation period. Their restitution following reinnervation probably depends upon the character of denervation changes in the muscle spindles at the time of onset of reinnervation.

This assumption is also supported by the investigation of muscles in 10-day-old animals after cutting the nerve at birth. The number of spindle remnants corresponded to the average number of atypical spindles found after reinnervation. The onset of reinnervation may be an important factor affecting the development of atypical spindles from spindle remnants. These spindles are localized near the nerve entry, evidently because regenerating nerve fibres reinnervate the region near the nerve entrance earlier than the rest of the muscle, and thus have a better chance of establishing a connexion with spindles prior to their disintegration.

However, the problem of the origin of atypical spindles cannot be conclusively solved without answering the question whether under normal conditions muscle spindles differentiate during muscle histogenesis only, or whether they may also be formed in adults. According to our results, this latter possibility seems to be improbable, because there is no significant change in the number of spindles in the two muscles studied in the rat from the tenth day of development up to the age of 5 months. On the other hand, some results relating to 10-month-old animals are considerably higher than the mean values found in the extensor digitorum longus of younger animals (Zelená & Hník, 1960). An increase in spindles during ageing was also described in the pectoralis muscle of the rat (Kalugina, 1956). The question therefore requires further investigation. In connexion with the abnormal structure of atypical spindles it will be necessary to study their innervation and functional characteristics as compared with normal spindles.

SUMMARY

1. The morphological characteristics of atypical spindles found in reinnervated rat muscles 5 months after crushing the sciatic nerve at birth were compared with normal control spindles.

2. The number of spindles found in the extensor digitorum longus and soleus was 32·9 and 12·7 spindles respectively, the relative number per decigramme being 24·8 spindles in the former and 13·1 in the latter. Spindles were found to contain 3·7 and 3·8 intrafusal fibres respectively. The length and diameter of spindles, the intrafusal fibre diameters, and the length of the nuclear bag did not differ markedly in the two muscles investigated. However, while the distribution and size of spindles in the soleus were fairly uniform, spindles in the extensor digitorum longus were restricted to the anterior half of the muscle only and the spectrum of their size was much wider.

3. The reinnervated muscles contained only occasional atypical spindles (0·55 spindles in the extensor digitorum longus and 1·5 in the soleus on the average). Their average length was approximately one-third to one-half of that of the normal spindles and they contained one or two intrafusal fibres. The diameter of the spindles and the intrafusal fibres is reduced and their structure resembles that of immature developing spindles.

4. In muscles 10 days after nerve section at birth, i.e. at the time of the onset of reinnervation, only occasional atrophic spindles were found. Their number corresponds closely to the number of atypical spindles found in reinnervated muscles 5 months later, so that it can be presumed that they probably originate from reinnervated spindle remnants which occasionally survive the transitory denervation period.

RÉSUMÉ

Les fuseaux musculaires atypiques dans les muscles réinnervés du Rat

1. On a comparé les caractéristiques morphologiques des fuseaux normaux témoins avec celles des fuseaux atypiques observés dans les muscles de Rat réinnervés, cinq mois après l'écrasement du nerf sciatique à la naissance.

2. Le nombre moyen de fuseaux trouvé dans les *extensor digitorum longus* et *soleus* était respectivement de 32,9 et 12,7, le nombre relatif par décigramme étant de 24,8 dans le premier cas et de 13,1 dans le second. Les fuseaux contenaient respectivement 3,7 et 3,8 fibres fusales. La longueur et le diamètre des fuseaux, les diamètres des fibres fusales et la longueur du sac nucléaire ne différaient pas de façon marquée chez les deux muscles étudiés. Néanmoins, alors que la répartition et la taille des fuseaux du *soleus* étaient assez uniformes, les fuseaux se trouvaient localisés à la moitié antérieure du muscle seulement chez l'*extensor digitorum longus*, et l'éventail de leurs tailles était beaucoup plus étalé.

3. Les muscles réinnervés contenaient seulement des fuseaux atypiques occasionnels (0,55 fuseau dans l'*extensor digitorum longus* et 1,5 dans le *soleus*,

en moyenne). Leur longueur moyenne était approximativement le tiers ou la moitié de celle des fuseaux normaux, et ils contenaient une ou deux fibres fusales. Le diamètre des fuseaux et des fibres fusales est réduit, et leur structure ressemble à celle de fuseaux immatures en cours de développement.

4. Dans les muscles pris 10 jours après la section des nerfs à la naissance, c'est-à-dire au début de la réinnervation, on n'a trouvé que des restes occasionnels de fuseaux. Leur nombre correspond étroitement à celui des fuseaux atypiques observés dans les muscles réinnervés cinq mois plus tard, de sorte qu'on peut présumer qu'ils proviennent de restes de fuseaux qui survivent parfois à la période transitoire de dénervation.

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EXPLANATION OF PLATE

Fig. 4. Normal spindle in extensor digitorum longus of adult rat. Cross-section through the equatorial zone. Two of the four intrafusal fibres exhibiting enlargement in the region of the nuclear bag. Supporting connective tissue strands partly disrupted following fixation.

Fig. 5. Two neighbouring spindles in close side-to-side contact with each other at the ends. Spindle above: myotubal zone. Spindle below: polar zone.

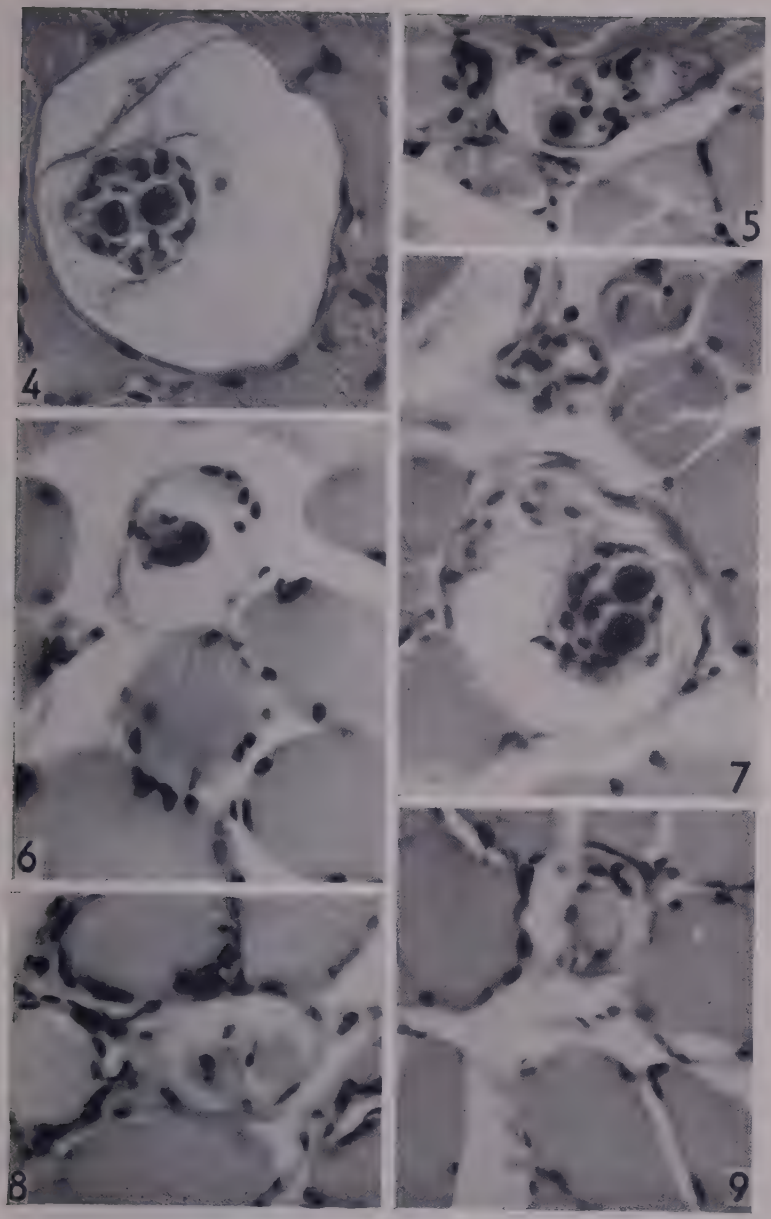

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FIG. 6. As in fig. 4. A section through the polar zone of a neighbouring spindle (top of figure).

FIG. 7. An atypical spindle in the extensor digitorum longus 5 months after crushing the sciatic nerve at birth. Note the small diameter of spindle and intrafusal fibres with under-developed nuclear bag.

FIGS. 8 and 9. Atypical spindles in reinnervated muscles of the rat containing abnormal intrafusal fibres with nuclei placed at their periphery.

FIGS. 4–9. Cross-sections of 20-μ thickness, stained with haematoxylin-eosin, $\times 500$.

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The Action of Various Agents upon the Rabbit Embryo

by C. E. ADAMS,[1] MARY F. HAY,[1] *and* CECILIA LUTWAK-MANN[2]

From the A.R.C. Unit of Reproductive Physiology and Biochemistry, and the Molteno Institute, University of Cambridge

WITH THREE PLATES

INTRODUCTION

In the course of an investigation on the uptake of labelled ions by the rabbit embryo and its environment (Lutwak-Mann, Boursnell, & Bennett, 1960), experiments were done on pre-implantation blastocysts obtained from rabbits which had been treated parenterally with certain purine analogues. Histological examination of such blastocysts by the method of Moog & Lutwak-Mann (1958) showed that, as a result of treatment of the pregnant animals, the embryos had incurred severe damage chiefly localized in the embryonic disc. This observation prompted a wider study, reported below, of the action of various agents upon the early rabbit embryo, following their administration to the mother. Our investigation was chiefly concerned with the pre-implantation $6\frac{1}{2}$-day-old blastocyst, but 5- and 7-day embryos were also examined. At the same time we have studied the influence of some of these agents upon ovulation, fertilization, and cleavage, as well as on implantation and later stages of pregnancy. We believe that these findings should be included as they contribute to a fuller understanding of the mode of action of embryotoxic factors.

The viability of cleaving eggs that had been placed for a few hours in the oviducts of rabbits under treatment with purine analogues, was also investigated; these eggs were subsequently transferred to normal permanent recipients, in which their development was studied at later stages of pregnancy (the egg-transfer experiments were carried out by C. E. A.). Some of our findings have already been briefly reported (Hay, Adams, & Lutwak-Mann, 1960).

[1] *Address*: Animal Research Station, 307 Huntingdon Road, Cambridge, U.K.

[2] *Address*: Molteno Institute of Biology and Parasitology, University of Cambridge, Downing Street, Cambridge, U.K.

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MATERIALS AND METHODS

Animals

The rabbits were bred and housed singly at the Animal Research Station; only does with an established record of fertility were used. The number of experimental rabbits was 200; 22 untreated animals provided control embryos.

Treatment of rabbits

The substances administered to the experimental animals were (*a*) hormones (oestradiol benzoate, diethylstilboestrol, dimethylstilboestrol, progesterone, desoxycorticosterone acetate, cortisone acetate); (*b*) antimitotic agents, comprising 2 colchicine derivatives (Colcemid, N-desacetylmethylcolchicine; Thiolcolciran, N-desacetylthiolcolchicine), and several polyfunctional alkylating agents (Thiotepa, triethylene-thiophosphoramide; TEM, triethylenemelamine; Degranol, β-bis-1,6-chlorethylamino-D-mannitol; E 39 soluble, 2:5-bis-ethylene-imino-3:6-bis-methoxyethoxybenzoquinone; Myleran, 1,4-dimethanesulphonoxybutane); 2 purine and 1 pyrimidine analogues (6-mercaptopurine; 8-azaguanine; 5-bromouracil); (*d*) vitamin A (special preparation of vitamin A acetate, containing 40,000 i.u. vitamin A per ml.); (*e*) vitamin antagonists (analogues of vitamin B_{12}, namely B_{12} methylamide and ethylamide, and B_{12} anilide; 2 folic acid antagonists, aminopterin and amethopterin); and (*f*) a group of miscellaneous agents (trypan blue; Neptazane, 2-acetylimino-3-methylthiadiazoline sulphonamide; carbon tetrachloride; carbutamide; cysteamine hydrochloride; X-radiation).

The timing of maternal treatment, and the dosage, represent a crucial but variable, experimental factor, and will be given in detail below. Unless otherwise stated, the agents were given parenterally. Irradiation with X-rays involved total body exposure. In this study the day of mating is referred to as day 0.

Histological technique

Pre-implantation blastocysts fixed in undiluted methanol were prepared as described by Moog & Lutwak-Mann (1958), except that the saline rinse was omitted. While the fixed blastocysts were in methanol, and before they were spread on to the cover-slips, their longest and shortest diameters, at right angles to each other, were measured by means of a square grid inserted into the eyepiece of the microscope. A Leitz screw-micrometer eyepiece was used to measure the embryonic disc of mounted, stained blastocysts; again, the longest and shortest diameters, at right angles to each other, were measured.

Mitotic counts were made in that portion of the abembryonic region of the blastocyst where the trophoblast consisted of only one layer of cells. The resting and dividing cells in 3–4 rectangular areas were counted in each 'arm' of the specimen, 450–650 cells usually being counted in each blastocyst. The mitotic index was expressed as the percentage of dividing cells in the total number counted.

Cleaving ova were examined by low-power microscopy; stages of pregnancy other than blastocysts, by direct visual observation.

Chemical methods

The activity of carbonic anhydrase was routinely assayed in the endometria of the treated animals during the progestational phase, by the method of Lutwak-Mann & Adams (1957). Determinations of bicarbonate, glucose, and lactic acid in the blastocyst fluid were made as previously described (Lutwak-Mann, 1954, 1960).

Egg transfer

Details of the procedure are described below (p. 481).

RESULTS

Experiments with untreated rabbits

Earlier observations on the appearance of normal 6-day blastocysts were extended to include embryos of 5 and 7 days so as to provide a wider basis for comparison with blastocysts from treated animals. The embryonic discs of blastocysts aged 5–7 days, were classified into 6 developmental stages (A–F), as set out in Table 1; stage A is the least mature, typical of day 5, and stage F

TABLE 1

Stages in the development of the embryonic discs in rabbit blastocysts at 5–7 days of gestation

Stage	*Description of embryonic disk*
A	Small disc with rather indefinite outline, composed of 1–2 layers of cells (Plate 1, fig. A)
B	Round disc with smooth outline and about 3 layers of cells thick (Plate 1, fig. B)
C	Disc beginning to elongate and grow out in a posterior direction, showing a slight thickening at its anterior edge (Plate 1, fig. C)
D	Onset of primitive-streak formation with cells beginning to condense in the midline of the zone of outgrowth (Plate 1, fig. D)
E	Primitive streak present in the posterior part of the disc.
F	Mesoderm beginning to grow out in a fan-shape at the posterior end of the disc (Plate 1, fig. E)

the most advanced, seen in some late 6-day, and the majority of 7-day, blastocysts (Plate 1, figs. A–E). When individual blastocysts were classified, intermediate stages were also recognized, e.g. in stage B–C the posterior growing edge of the disc could be distinguished, although it had not yet begun to grow out.

The diameters of entire unopened blastocysts, and of the embryonic discs, were measured in 133 embryos aged 5–6$\frac{2}{3}$ days and taken from 17 litters; the

mean diameters for each litter are recorded in Table 2. So far as one could tell from direct visual observation, fixation in methanol induced no appreciable change in either shape or size of the blastocysts. On day 5 the embryos were almost spheroidal, the mean diameters of entire blastocysts ranging from 1·3 × 1·3 mm. to 2·2 × 2·1 mm., and those of the discs from 0·64 × 0·59 to 0·90 × 0·77 mm. During the 6th day the embryos became ellipsoidal in shape, the mean entire diameters ranging from 2·2 × 2·2 mm. to 4·8 × 4·4 mm., and the discs from 1·0 × 0·85 mm. to 1·35 × 0·99 mm. Only two 7-day blastocysts were measured, and their embryonic discs were not substantially larger than those of the bigger 6⅔-day embryos. Table 2 also shows the percentage number of blastocysts in each stage in 19 normal litters examined on days 5–7; the litters are arranged according to the mean diameters of the entire blastocysts. It can be seen that the first sign of primitive streak formation (stage D) was not reached until the litter had a mean blastocyst diameter of over 4 mm.; this was also true for individual blastocysts. However, blastocysts of over 4 mm. often had discs in stage C.

TABLE 2

Size of entire blastocyst and of embryonic disc in relation to developmental stages A–F

Age and no. of blastocysts examined in each litter	*Mean diameters of entire blastocysts in mm.*		*Mean diameters of embryonic discs in mm.*		*Number of blastocysts (%) in stage*										
					A	*A–B*	*B*	*B–C*	*C*	*C–D*	*D*	*D–E*	*E*	*E–F*	*F*
Day 5															
6	1·3	1·3	0·64	0·59	67	33	..	..	..	..	..	..	..	..	..
8	1·5	1·4	0·60	0·52	88	12	..	..	..	..	..	..	..	..	..
4	1·9	1·8	0·78	0·65	50	25	25	..	..	..	..	..	..	..	..
10	2·2	2·1	0·90	0·77	10	10	80	..	..	..	..	..	..	..	..
Day 6															
10	2·2	2·2	1·00	0·85	..	40	50	10	..	..	..	..	..	..	.
9	2·9	2·8	1·21	1·10	..	22	56	22	..	..	..	..	..	..	..
9	3·2	3·0	0·99	0·92	..	11	23	33	33	..	..	..	..	..	..
8	3·4	3·2	1·05	0·96	12	..	25	..	63	..	..	..	..	..	..
5	3·4	3·2	1·06	0·99	..	..	40	60	..	..	..	..	..	..	..
10	3·6	3·5	1·16	1·06	..	..	30	40	30	..	..	..	..	..	..
6	3·8	3·6	1·13	1·03	..	..	17	16	67	..	..	..	..	..	..
7	3·9	3·5	1·12	1·01	..	..	14	14	72	..	..	..	..	..	..
13	4·4	4·1	1·17	0·99	..	..	8	7	23	8	15	8	31	..	..
7	4·5	4·0	1·09	1·05	..	..	..	14	86	..	..	..	..	..	..
10	4·5	4·2	1·28	1·02	..	..	10	..	10	..	10	20	50	..	..
11	4·8	4·4	1·18	1·01	..	..	9	9	27	..	18	9	28	..	..
5	4·8	4·2	1·35	0·99	..	..	..	..	..	20	..	..	20	..	60
Day 7															
3	..	..	1·32	1·01	..	..	..	..	..	..	..	..	33	..	67
5	..	..	1·33	0·98	..	..	..	..	..	20	..	..	..	20	60

The embryonic discs of only two 7-day blastocysts were measured, one in each litter.

Trophoblastic knobs, described by Schoenfeld (1903), were found in some of the larger 6-day and in 7-day embryos in the double-layered part of the abembryonic hemisphere, close to the edge of the endoderm (Plate 2, fig. F). Often a few

knobs were found together while the rest of the trophoblast was devoid of them. No knobs were seen in blastocysts with a diameter below 4 mm., but there were somewhat larger blastocysts in which no knobs were present. The development of the knobs did not appear to be closely correlated with that of the embryonic disc; they were not associated with discs earlier than stage C, though some embryos in stage F had no knobs.

Nuclear degeneration was commonly observed in the embryonic area, and a moderate amount of it was considered normal (see Glücksmann, 1951). The distribution of the degeneration granules was characteristic for each stage of development, e.g. they were distributed throughout the stage B discs, but were concentrated in their anterior parts in stage C. Globules of material which stained metachromatically with haematoxylin were frequently found associated with the degenerating cells. This material formed small nodules projecting into the blastocyst cavity, stained positively with Alcian blue, and gave the periodic acid Schiff reaction; it seems likely therefore that the material was mucin. These nodules could be seen in unstained methanol-fixed blastocysts, where they appeared black in transmitted light, and frequently also in fresh embryos. The presence of mucin located in the disc area appeared to be normal in 5- to 7-day blastocysts, though it was not a constant finding. Usually, mucin was present either in all the members of a litter, or in none. Isolated globules of mucin were sometimes seen in the trophoblast.

Effect of hormones

The experiments with hormones were largely confined to the blastocyst stage and the period of implantation.

Oestradiol benzoate

When given in doses of 0·5–1·0 mg. per animal, on days 4–5 after mating, this hormone induced marked uterine hypertrophy; often there was pyometra, or if this was absent, there was abundant uterine secretion, and the endometrium was oedematous and haemorrhagic. Blastocyst recovery was impeded by pus, and the embryos showed signs of degeneration and infection. However, in pus-free uteri, well-developed 6½-day blastocysts were usually recovered, and some appeared to be embedding prematurely. In two experiments the primitive streak had formed in 40–50 per cent. of the embryos.

Following treatment of rabbits with oestradiol benzoate, on days 4 to 5 of gestation, implantation seldom succeeded, presumably owing to the grossly altered uterine environment, and not because of any primary damage to the embryos themselves.

Diethylstilboestrol

This was given in doses of 0·5–5·0 mg. per animal, on days 4–5 after mating. Unlike with oestradiol, pyometra was infrequent, but the uteri were enlarged and

contained an excessive amount of uterine secretion. In one experiment over 50 per cent. of the 6½-day blastocysts showed signs of primitive-streak formation, and were already attached to the oedematous endometrium; in another, blastocysts of the same embryonic age were small (diameter 2·7 × 2·3 mm.) and the disks in an early stage of development, none being more advanced than stage B–C.

Stilboestrol given at 4–5 days, did not prevent blastocyst implantation. However, foetal development did not continue beyond 12–14 days, presumably owing to the profound changes in the uterine tissues.

Dimethylstilboestrol

Unlike diethylstilboestrol, this stilboestrol derivative given to rabbits in doses of 0·1–5·0 mg. per animal, on days 4–5, caused little hypertrophy in either the myometrium or the endometrium. The 6½-day blastocysts appeared normal on microscopical examination. Nevertheless, pregnancy terminated a few days after implantation.

Progesterone

There was no stimulating or other discernible effect upon the 6½-day blastocysts, when progesterone (1 mg. per animal) was given daily on days 1–4.

Desoxycorticosterone acetate

This hormone probably had no effect upon 6½-day blastocysts following treatment of rabbits with less than 5 mg. per animal daily, starting on days 1, 2, or 3; with 5-mg. doses some embryos showed signs of localized degeneration in the trophoblast. Implantation was normal when treatment did not exceed 1–2 mg. daily.

Cortisone acetate

Normal 6½-day blastocysts were recovered from rabbits injected daily with 12 mg. per animal, beginning on day 0 or 1, and continuing until day 5. The treatment did not interfere with the progress of pregnancy.

Effect of colchicine derivatives

Colcemid

Rabbits given single injections of Colcemid ranging from 0·3 to 8·0 m.g. per kg. body-weight developed diarrhoea and lost weight. These symptoms were transient with doses up to 6 mg. per kg.

Ovulation and fertilization. There was no interference with either function within the above dose-range. Similarly, does that had been treated with small amounts of Colcemid on alternate days for 3 weeks were capable of mating and conception.

Cleavage. The administration of 2–5 mg. per kg. caused arrest of ovum cleavage within a few hours of the injection.

Blastocyst stage. Blastocysts were examined at $5\frac{1}{2}$, $6\frac{1}{2}$, and $6\frac{2}{3}$ days of age, after long- and short-term experiments. In the former, a single dose of Colcemid was given on days 1, 2, 4, or 5, and the blastocysts were examined at $6\frac{1}{2}$ days. In the latter, the animals were injected either on day 5 or on day 6, and autopsied 1, 5, 9, or 14 hours later.

The effect of this drug on the histology of the blastocysts was related both to the time of administration and the dose level. In long-term experiments, 1 mg. per kg. or less, given on day 1 or 2, had no visible effect at $6\frac{1}{2}$ days. The same dose, given on day 4 or 5, had an inhibitory effect on the discs, none of which had reached a stage beyond B–C, by $6\frac{1}{2}$ days. The injection of 2 mg. per kg. on day 5, 36 hours before autopsy, had a marked deleterious effect. The blastocysts were small, some had collapsed, and in all the discs were irregular and degenerating, while the trophoblasts also showed signs of damage. In some instances there were numerous lymphocytes on the outer surface of the blastocysts.

TABLE 3

Mitotic activity in the abembryonic area of the trophoblast in blastocysts from normal and Colcemid-treated rabbits

Dose of Colcemid (mg./kg. body-wt.)	*Interval between injection and autopsy in hours*	*Age of blastocysts at autopsy (days after mating)*	*No. of blastocysts on which counts were made*	*Total no. of cells counted*	*Cells in mitosis*	
					Number	%
..	..	$5\frac{1}{2}$	4	2,310	110	4·8
..	..	$5\frac{2}{3}$	4	1,629	56	3·4
..	..	6	5	2,246	152	6·8
..	..	6	4	2,281	91	4·0
..	..	$6\frac{1}{2}$	3	1,853	58	3·1
..	..	$6\frac{1}{2}$	4	2,460	88	3·6
8	36	$6\frac{1}{2}$	4	2,533	68	2·7
7	36	$6\frac{1}{2}$	3	1,787	63	3·5
2	14	6	4	2,626	157	6·0
5	9	$6\frac{2}{3}$	5	2,597	1,668	64·2
2	9	$6\frac{2}{3}$	4	1,802	1,245	69·1
5	5	$6\frac{2}{3}$	3	1,718	476	27·7
5	5	$6\frac{2}{3}$	4	1,855	652	35·1
5	5	$6\frac{2}{3}$	5	2,619	483	18·4
2	5	$6\frac{1}{2}$	5	3,138	645	20·6
2	5	$5\frac{1}{2}$	3	718	333	46·6
2	1	$6\frac{1}{2}$	4	2,229	189	8·5
2	1	$6\frac{1}{2}$	4	2,493	194	7·8

When Colcemid was given on day 6, at 1, 5, or 9 hours before autopsy, the characteristic antimitotic activity of the drug was clearly revealed: cell-division in both disc and trophoblast was arrested in metaphase (Plate 2, figs. G, H), and as a result no anaphases were seen. Cells continued to attempt division, however, and prophases were present in all experiments. It can be seen from Table 3, in

which each experiment refers to one litter, that in the majority of $5\frac{1}{2}$- to $6\frac{1}{2}$-day blastocysts from untreated control rabbits less than 5 per cent. of the cells in the abembryonic area of the trophoblast, in which cell counts were made, were dividing. In blastocysts examined 1 hour after injection of 2 mg. per kg. Colcemid, 7–8 per cent. of the trophoblastic cells were in division, the majority in metaphase, but late telophases were seen in some blastocysts, presumably representing the end of the last cycle before the mitotic block had become effective. With a 5-hour interval between injection and autopsy, the percentage of dividing cells was 18–35, and when the interval was 9 hours, the percentage was 64–69. In the last two groups of experiments, again the majority of dividing cells was in metaphase but, in contrast to the embryos examined 1 hour after injection, the only other stage seen was prophase.

Blastocysts examined on day 6, 14 hours after injection, showed no signs of metaphase arrest; 6 per cent. of the abembryonic cells were in division, and some multinucleate cells were present, probably resulting from the earlier disturbance in mitotic activity. The block to spindle formation and anaphase appeared to wear off during the 9–14 hours' interval after treatment, allowing the cells to complete their division. Results were essentially similar when short-term experiments were started on day 5; 47 per cent. of the cells in the abembryonic area of the trophoblast were in prophase or metaphase 5 hours after Colcemid injection.

Treatment with Colcemid also resulted in marked cellular degeneration apparent in the discs 5 hours after injection (Plate 2, fig. G). In the trophoblasts, this was not obvious until 14 hours after administration of Colcemid. The degeneration was still visible 36 hours after treatment, long after normal mitotic activity had presumably been resumed. Growth and differentiation of the discs was suppressed; the mean diameters of discs at $6\frac{1}{2}$–$6\frac{2}{3}$ days, 5 and 9 hours after treatment, were $0{\cdot}60 \times 0{\cdot}57$ and $0{\cdot}71 \times 0{\cdot}63$ mm., respectively; none had developed beyond stage B. The size of the entire blastocysts varied considerably but was within normal limits in the majority of the embryos.

Implantation and later stages of pregnancy. In rabbits that had received 2 mg. per kg. Colcemid on day 6 and were autopsied 1–2 days later, about 50 per cent. of the embryos were implanted, but the rest were lying free; the implantation domes and the unattached blastocysts were smaller than normal. There were no obvious changes in the uterine tissues. Chemical analysis of the blastocyst fluid from the $7\frac{1}{2}$-day embryos which failed to implant showed that they were retarded in their development; this was indicated by a high content of bicarbonate (150 ml. CO_2/100 ml.), and low values for glucose (15 mg./100 ml.) and lactate (38 mg./100 ml.), such as are typical of 6-day blastocysts (Lutwak-Mann, 1960).

Rabbits given 0·5 mg. per kg. on alternate days for 3 weeks before mating carried their litters to term. At birth the young were rather small but there were no obvious malformations. In spite of apparent good maternal health, the litters did not survive; this was thought to be due to failure of lactation. The

does were then mated again; they conceived and this time successfully reared their offspring.

Thiolcolciran

Rabbits were given injections of 2–12 mg. per kg. body-weight which were followed by transient diarrhoea and a fall in weight. The impression was gained that this colchicine derivative was less toxic to rabbits than Colcemid.

Ovulation and fertilization. These were not affected by the doses used.

Cleavage. Cell-division was rapidly arrested after the administration of 5–10 mg. per kg.; smaller amounts were not tested.

Blastocyst stage. The response elicited in blastocysts depended on the time of treatment. A small dose given on day 4 allowed the formation of some apparently normal blastocysts, but numerically the litter was reduced in comparison with previous pregnancies. Treatment with 8–10 mg. per kg. on day 5 resulted in blastocysts which at $6\frac{1}{2}$ days were normal in size (diameter of $3{\cdot}5 \times 3{\cdot}2$ mm.) but had very small discs ($0{\cdot}63 \times 0{\cdot}53$ mm.), none of which had progressed beyond stage B. Injection of 4–6 mg. per kg. on day 6, followed by autopsy 9 hours later, caused the appearance of numerous small degeneration granules in the disc, and some degeneration in the trophoblast. In contrast to experiments with Colcemid, no upset in mitotic activity was observed.

Implantation. Blastocyst nidation was largely prevented by 8–12 mg. per kg. given on days $5\frac{1}{2}$–6. The effect on later stages of gestation was not studied.

Effect of polyfunctional alkylating agents

Thiotepa

This was given parenterally in doses of 3–10 mg. per kg. body-weight. The larger amounts caused a marked loss of weight, but there were no other overt symptoms in experiments of limited duration.

Ovulation and fertilization. These were not affected by doses up to 6 mg. per kg.; with higher doses some 25 per cent. of the ova shed were unfertilized.

Cleavage. Early arrest of cleavage invariably occurred, even in response to small doses.

Blastocyst stage. Thiotepa given on days 3, 4, 5, or 6 affected all blastocysts adversely, though there were differences in the susceptibility of the litters. The histological appearance of the $6\frac{1}{2}$-day embryos depended on the timing of the treatment, the earlier it was given, the more drastic the effect. When the interval between injection and autopsy was only 9 hours, the disc was full of degeneration granules but its stage of development could still be recognized, the majority of embryos being in stage C. Following treatment on day 5, the $6\frac{1}{2}$-day disc had lost its general organization, and degeneration granules were still present (Plate 2, fig. I). After treatment on day 3 or 4, only a very small thin disc containing a few degeneration granules and sometimes mucin, remained. The effect of Thiotepa seemed to be exerted mainly on the disc, but the trophoblast was

probably also affected, as blastocysts were small (diameter 2–3 mm.) at $6\frac{1}{2}$ days, following treatment on days 3 and 4. No changes in mitotic activity were visible in the embryos at the time of autopsy.

Implantation and later stages of gestation. No experiments were done to examine the effect on nidation as such. However, even low doses of Thiotepa prevented gestation in rabbits, none of which produced living offspring following treatment on days 6–8 with 3 mg. per kg.

Triethylenemelamine (TEM)

This substance caused a marked fall in weight, even in doses of 0·5 mg. per kg. body-weight; no other symptoms were seen in experiments of short duration.

Ovulation and fertilization. Single doses not exceeding 2 mg. per kg. given on day 0, or a series of 12 injections of 0·5 mg. per kg. given over a period of 24 days prior to mating, had no effect on ovulation. Following 2 mg. per kg. given immediately after mating, 25 per cent. of the ova shed were unfertilized.

Cleavage. Doses of 1·0–2·0 mg. per kg. rapidly inhibited ovum cleavage.

Blastocyst stage. A single dose of 0·5–2·0 mg. per kg. given on days 2, 4, 5, or 6 had a drastic effect on the blastocysts, the response generally being the more marked the earlier the drug was given.

Usually only debris of cleaving ova or morulae were found at 6 days in the uteri of animals treated soon after mating. In one experiment, following a small dose on day 0, one apparently normal blastocyst was recovered, while the rest of the embryos were completely degenerated. The rate of growth of the embryos recoverable at 6 days was slowed down: $6\frac{1}{2}$-day blastocysts from does treated on day 4 were smaller ($3{\cdot}0 \times 2{\cdot}9$ mm.) than those from an animal injected on day 5 ($4{\cdot}0 \times 3{\cdot}7$ mm.). Histological changes, dependant upon the time of treatment, were seen in the discs of $6\frac{1}{2}$-day embryos. When the experimental interval was limited to 9 hours, marked signs of degeneration were present. Following treatment on day 5, the $6\frac{1}{2}$-day discs were small and thin, and the cells contained distinct, heavily stained granules (Plate 2, fig. J)—probably chromatin, a picture resembling that produced by Thiotepa given on day 5. After TEM injection on day 4, the $6\frac{1}{2}$-day discs were very small and contained mucin and large extracellular granules (Plate 2, fig. K). Although there was some degeneration in the trophoblast, there were many dividing cells there, and mitosis was not obviously affected.

Implantation and later stages of pregnancy. A low dose of TEM on day 6 was incapable of preventing the attachment of blastocysts, but the further development of such embryos usually terminated within a few days. By treating rabbits with not more than 0·5–1·0 mg. per kg. on day 8, i.e. after implantation, it was possible to maintain pregnancy till term, but the young were born dead; malformations were not seen. The does were again mated and conceived normally. However, by about day 20 of gestation massive vaginal haemorrhage abruptly terminated the pregnancies. Inspection of the reproductive tract did not reveal

abnormalities, apart from the haemorrhagic residue. In this respect, that is in being apparently able to induce discrete yet long-term changes in the reproductive system of rabbits, TEM differed from the other agents used in this study.

Degranol

This nitrogen mustard derivative was given intravenously in doses of 12–45 mg. per kg. body-weight. The lower dose was well tolerated; the larger amount caused some loss of weight, but the rabbits survived for another 10 days, when the experiments were terminated.

Ovulation and fertilization. Neither was affected at the 12-mg. per-kg. dose level; higher doses were not tested.

Cleavage. No experiments *in vivo* were done; *in vitro*, the development of 2- to 4-cell rabbit ova was arrested after a few hours' incubation in serum-Ringer solution containing Degranol 1:10,000.

Blastocyst stage. Normal blastocysts were found at $6\frac{1}{2}$ days in one rabbit given 12 mg. per kg. on days 0 and 1; some of the embryos of another animal given 45 mg. per kg. on day 5 were so fragile that they burst before they could be fixed in methanol. This did not occur with any other treatment and was probably the result of an adverse effect upon the trophoblast or the zona pellucida. The remainder of these blastocysts all showed degenerative changes in various degrees, in both the discs and trophoblasts.

Implantation and later stages of pregnancy. Blastocyst nidation was not prevented by 12 mg. per kg. given on day 6; a normal litter was born to a doe injected with this dose on day 8. In a rabbit given 38 mg. per kg. on day 8, small implants were found on day 12; several of these were degenerating, while some were still alive. The foetal placentae were strikingly pale and poorly developed, and the exocoelomic fluid was scarce and contained stringy masses.

E 39 soluble

This was given as a single subcutaneous injection of 2·0–2·8 mg. per kg. body-weight on day 4 or 5. The dose was well tolerated and there was no local reaction. So far, the effect of this drug has been studied on blastocysts only, on which it had a severe destructive action. In some animals 50 per cent. of the embryos were almost completely destroyed. The blastocysts which survived to day $6\frac{1}{2}$ were small (mean diameters less than 3 mm.); there was much degeneration in the discs, and rather less in the trophoblasts, the embryos from mothers treated earlier being more seriously affected (Plate 3, figs. L, M). There appeared to be some effect on mitosis as some abnormal mitotic figures were seen.

Myleran

Doses of 4·8–12 mg. per kg. of body-weight given parenterally or orally, on days 1 or 5, or 4 and 5, had no effect on $6\frac{1}{2}$-day blastocysts.

Effect of purine and pyrimidine analogues

6-Mercaptopurine

This was injected subcutaneously in doses ranging from 25 to 250 mg. per kg. of body-weight. Rabbits were remarkably resistent to mercaptopurine and withstood even large amounts well. In most experiments on the pre-implantation blastocysts the injections were made 24–48 hours before autopsy, but in others they were given on day 0 or 1.

Ovulation and fertilization. There was no interference with either within the dose-range.

Cleavage. Tubal ova examined on days 1, 2, or 3, following administration of mercaptopurine 20–24 hours earlier, showed no detectable abnormalities on microscopical examination. Moreover, such ova, when recovered on day 1 or 2 and subsequently cultured *in vitro* in serum-Ringer solution, continued to divide normally for another 24 hours. Nevertheless, as will be demonstrated below (p. 481), the majority of such ova would have been incapable of normal full-term development.

Blastocyst stage. Blastocysts were examined at 6–$7\frac{3}{4}$ days of age. Their size and gross appearance at 6–$6\frac{1}{2}$ days was not perceptibly altered following treatment of rabbits shortly after mating or on days 4–5. However, at 7–$7\frac{3}{4}$ days, when normally rabbit blastocysts are already attached to the uterus, most embryos from mercaptopurine-treated rabbits had failed to implant although they continued to expand in size. Such 'giant' free-lying blastocysts often reached a diameter of 5 mm., the maximum size recorded being $7{\cdot}6 \times 6{\cdot}1$ mm., and weighed up to 100 mg. Occasionally at that stage the uterine horns of the treated animals were filled with a water-clear, alkaline fluid, in which, on microscopical examination, blastocyst debris could be seen. Chemical analysis showed that this fluid contained bicarbonate, glucose, and lactic acid in amounts corresponding to those established for 6- to $6\frac{1}{2}$-day blastocyst fluid.

Histological study of these blastocysts indicated that mercaptopurine exerted a strongly deleterious effect on the embryos, whether administered to the mother shortly after mating, or on days 4–5. The embryonic disc had almost disappeared in some blastocysts treated on day 0 or 1, while in others it consisted of a few cells with darkly staining cytoplasm (Plate 3, fig. N) surrounding a mass of reddish globular material, believed to be mucin. In blastocysts from animals treated on days 4–5 (Plate 3, fig. O) there were more degeneration granules, but less mucin, than in those injected soon after mating (Plate 3, fig. N). The trophoblasts were relatively less affected than the disks and appeared almost normal in some embryos. In many but not all of the 7- to $7\frac{3}{4}$-day blastocysts, the development of the trophoblastic knobs was either reduced or prevented.

Later stages of pregnancy. In rabbits treated as indicated above a few blastocysts implanted, but foetal development usually terminated between days 12 and 16; on autopsy, the foetuses were undersized and in various stages of

degeneration. The maternal and foetal placentae were regressing. No living young were born to rabbits that at any time after mating had received more than 75 mg. per kg. mercaptopurine.

8-Azaguanine

This was injected in doses 25–165 mg. per kg. on 1 or 2 of the first 5 days after mating. The health of the animals remained good.

Ovulation and fertilization. There was no interference with ovulation when azaguanine was given immediately after mating, but 10–15 per cent. of the ova shed were unfertilized when the dose was large.

Cleavage. Following treatment on days 0, 1, or 2 it was not possible to detect any morphological changes in cleaving ova recovered 24 hours after the injection. Yet these ova must have incurred discrete damage, as can be inferred from the egg-transfer experiments.

Blastocyst stage. Following treatment of rabbits on days 0, 1, or 2 and sometimes 3, blastocysts were seldom found at $6\frac{1}{2}$ days, but on flushing the uterine horns with saline it was possible to recover remnants of degenerating morulae or even early blastocysts. In one experiment, with injection on day 3, small blastocysts (diameter less than 1·7 mm.) were recovered on day $6\frac{2}{3}$. These embryos were severely damaged: the embryonic disc had almost disappeared and consisted of a few degenerating cells, but there was less mucin than in similar experiments with mercaptopurine. On the other hand, apparently normal blastocysts were observed following treatment on day 4 or 5. The impression was gained that with azaguanine the lesions in the embryos developed slowly, so that unless enough time was allowed to elapse between the time of administration and autopsy, the extent of damage appeared relatively slight. Possibly also, rabbit embryos were more sensitive to this purine analogue in the early, rather than in the slightly more advanced, stages of development.

When 25 mg. per kg. azaguanine was injected with an equal amount of mercaptopurine on days 4 and 5, very small blastocysts (mean diameters 2·2 × 2·1 mm.) were recovered at $6\frac{1}{2}$ days. All embryos showed marked signs of degeneration in the disc while the trophoblast had suffered less damage. The combined effect of the two purine analogues seemed more severe than that of a similarly timed dose of 50 mg. per kg. mercaptopurine alone. There were no changes in the uterine tissues, upon visual examination, with either of these substances.

5-Bromouracil

A limited number of experiments was done with this pyrimidine analogue. Doses of 50–80 mg. per kg. administered on days 2 and 3 had no deleterious effect on $6\frac{1}{2}$-day blastocysts as regards size and microscopical appearance. Rabbits similarly treated but allowed to go to term produced litters of normal

size which, however, failed to survive although maternal health was not noticeably affected.

Embryonic mortality following temporary transfer of cleaving eggs to the oviducts of rabbits treated with purine analogues

Egg-transfer technique

Eggs were recovered from the oviducts of 12 donor rabbits which had been mated 24–26 hours earlier with 2 fertile males. Some of the eggs were incubated *in vitro* in Krebs's Ringer solution+homologous blood plasma (1:1) at 37° C. for 4½–6 hours. These eggs are referred to as 'controls'. Other eggs were transferred for 4½–6 hours into the oviducts of rabbits serving as 'temporary recipients'; these animals were given one injection of either mercaptopurine or azaguanine (110–180 mg. per kg.) on the evening preceding, and another on the day of, the experiment. Such eggs, referred to as 'treated', were next recovered for transfer into the oviducts of untreated permanent recipients; the luteal stage of these animals (in terms of hours after injection of luteinizing hormone) was arranged to correspond with the age of the transferred ova. The 'treated' eggs were introduced on one, the 'control' eggs on the other side of the reproductive tract of the permanent recipients. The further development of the transplanted embryos was examined by laparotomy or autopsy between days 9–14 of gestation; 3 does were allowed to go almost to term.

Results

The 40 'control' eggs underwent one cleavage division, reaching the 4-cell stage during incubation *in vitro*. Out of the 70 eggs placed in the oviducts of the 'temporary recipients', 3 of which were under treatment with mercaptopurine and 3 with azaguanine, 57 were recovered. With few exceptions, all had undergone one cleavage division. Morphologically, the 'treated' eggs appeared indistinguishable from the 'controls'.

Following temporary exposure of 21 eggs to the tubal environment of rabbits treated with mercaptopurine, the implantation rate was 23·8 per cent. and that for 17 'control' eggs 70·6 per cent. A high proportion of foetuses from the 'treated' eggs showed retarded growth at autopsy on day 12; these probably would not have survived to term. In one recipient which had received 9 'treated' eggs a single, apparently normal, foetus was found at 28 days.

In analogous experiments with azaguanine, some 60 per cent. of the 20 'treated' eggs were found implanted at 10–14 days, but several implantation sites were poorly developed, and in some the embryos were dead. One doe which went to term had 2 apparently normal foetuses from 6 'treated' eggs. In contrast, all foetuses from the 11 'control' eggs in these experiments were normally developed.

Though restricted in number, the results indicate that the deleterious effect of mercaptopurine and azaguanine can be transmitted to the cleaving egg during

its stay in the oviduct, and that a relatively brief exposure *in vivo* suffices to induce a high percentage of embryonic loss. It is noteworthy, and in line with observations recorded earlier (pp. 479 and 480), that the *in vivo* effect of the purine analogues was not immediately apparent, but that it became manifest at about the time of, or after, ovum implantation.

Effect of excess vitamin A

The vitamin A preparation was fed or injected between day 0 and 5; the total varied from 60,000–300,000 i.u. per animal. The treatment had no effect upon the health of the rabbits.

Blastocyst stage. There was no evidence of any deleterious effect of excess vitamin A upon $6\frac{1}{2}$-day blastocysts; indeed, some embryos were larger than normal, and in one litter all showed signs of primitive-streak formation (stage D or beyond), a well-formed streak being present in 57 per cent.

Implantation and later stages of pregnancy. There was no interference with either nidation or subsequent foetal development, when treatment with excessive amounts of vitamin A was restricted to days 0–5. Normal litters born to treated does were reared and in turn produced normal offspring.

Effect of vitamin antagonists

Analogues of vitamin B_{12}

B_{12} anilide alone, or a mixture of B_{12} methylamide and ethylamide (Smith, Parker, & Gant, 1956) were injected daily in doses of 100–500 ug. per animal, starting 10 days before mating and continuing throughout pregnancy and lactation. The animals' health remained excellent.

Blastocyst stage. There was no demonstrable effect upon $6\frac{1}{2}$-day blastocysts.

Implantation and later stages of pregnancy. Normal litters were born to all treated rabbits.

Analogues of folic acid

Aminopterin and amethopterin were the folic acid antagonists used. Only the effect upon blastocysts was investigated.

Aminopterin. Given parenterally during the pre-implantation period, 1–2 mg. per kg. had no marked deleterious effect upon rabbits. Blastocysts recovered at $6\frac{1}{2}$–$6\frac{2}{3}$ days from rabbits given 1 mg. per kg. on day 4 were small (diameter 2·6 × 2·4 mm.) and the discs were immature: the most advanced stage was B–C, in which were 43 per cent. of the discs. However, after similar treatment on day 5, $6\frac{1}{2}$-day blastocysts appeared normal. When 2 mg. per kg. was given on day 6 and followed by autopsy 9 hours later, the embryos were normal; they showed no disturbance of mitotic activity.

Amethopterin. This was administered parenterally or orally. Injections of up to 6·5 mg. per kg. on days 0, 2, or 3, or oral doses of 5 mg. per kg. on days 0–5, did not visibly impair the health of the rabbits. There were no visible effects upon $6\frac{1}{2}$-day embryos with either type of treatment.

Miscellaneous agents

Trypan blue

A 10 per cent. solution of the dye in saline was injected either on days 0–5, or 7–9, in doses of 100–200 mg. per kg. It exerted no ill-effects on the rabbits.

Blastocyst stage. Trypan blue administered within the first 5 days after mating failed to penetrate unimplanted blastocysts, which remained colourless, although the uterine secretion and the endometrium were strongly stained. Microscopically these embryos were normal. Attempts were made to increase the permeability of pre-nidation blastocysts by combining trypan blue treatment with a single small dose of TEM or Colcemid given on day 5. Even then, however, the dye did not enter the blastocysts.

Implantation and later stages of pregnancy. The negative results in the pre-implantation embryos contrasted with the effect of trypan blue when it was injected during the implantation period, i.e. on days 7–9. At that stage the dye freely entered the blastocyst cavity; a large proportion of young born to rabbits treated in this way showed malformations; none survived beyond a few days.

Neptazane

This potent sulphonamide inhibitor of carbonic anhydrase was used to study the structure and chemical composition of blastocysts developing under conditions in which the activity of endometrial carbonic anhydrase was greatly curtailed. Neptazane was given intravenously, 100–120 mg. per kg. body-weight, in subdivided doses, on days 4, 5, and 6 without causing any toxic symptoms. Following such treatment the activity of blood carbonic anhydrase was almost abolished; however, some residual enzyme activity invariably remained in the endometrium (20–25 e.u. per g.).

Blastocyst stage. No abnormal structural changes were observed in $6\frac{1}{2}$-day embryos following exhaustive treatment with Neptazane. The content of bicarbonate, glucose, and lactate in the blastocyst fluid at 6–8 days did not differ from values established for normal blastocysts.

Implantation and later stages of pregnancy. There was no interference with nidation. Later foetal development was normal.

Carbon tetrachloride

This hepatotoxic substance was fed to pregnant rabbits in arachis oil, 0·6 ml. per kg. body-weight on day 5 or 1·0 ml. per kg. on days 4 and 5. The treatment caused no overt symptoms in these short term experiments. Blastocysts recovered at $6\frac{1}{2}$ days were well developed and appeared quite normal after treatment with the smaller dose. With the larger amount there was some cellular degeneration in the embryonic discs, and the trophoblasts of some embryos contained very large nuclei with prominent nucleoli.

Carbutamide

This hypoglycaemia-inducing agent was administered parenterally, in doses 100 mg. per kg. body-weight and was well tolerated.

Blastocyst stage. Following treatment on days 4, 5, and 6 no abnormalities were seen in $6\frac{1}{2}$-day blastocysts.

Implantation and later stages of pregnancy. There was no interference with foetal development; a normal litter was born to a treated doe.

Cysteamine hydrochloride

Doses of this radiation-protective agent ranging from 70 to 160 mg. per kg. body-weight were well tolerated.

Blastocyst stage. Following injection on days 2 and 3, 3 and 4, or $4\frac{1}{2}$ and 5, blastocysts recovered at $6\frac{1}{2}$–$6\frac{2}{3}$ days appeared unaffected by the treatment.

Implantation and later stages of pregnancy. There was no interference with nidation or further foetal development.

Total body exposure to X-rays

Pregnant rabbits were exposed to X-radiation ranging from 450–650 r., given at a dose-rate of 150 r. per minute. The Maximar X-ray machine was run at 220 kV., 15 Ma. and an added 1 mm. aluminium filter was used. The unanaesthetized animals were confined in a lidless wooden box, in such a way as to receive the radiation beam mainly in the lumbar region, the distance from the animal's body to the source of radiation being 20 cm. There were no overt symptoms following this type of exposure within the limited experimental period.

Two rabbits were treated on day 5 with 450 r. and 650 r., respectively, and the blastocysts were fixed 36–40 hours later; two other animals were similarly irradiated on day 6 and autopsied 5 and 9 hours later. Following irradiation on day 5 with 450 r., the development of the disc appeared retarded, the most advanced stage at $6\frac{2}{3}$ days being C, but the mean diameter of the blastocysts was normal ($4{\cdot}2 \times 3{\cdot}9$ mm.) and 70 per cent. of them had trophoblastic knobs. There was some nuclear degeneration but this was probably not excessive. Similarly timed treatment with 650 r. resulted in small blastocysts ($3{\cdot}1 \times 2{\cdot}9$ mm.); at $6\frac{1}{4}$ days none was more advanced than stage B. In this experiment degeneration granules were rather numerous. The blastocysts recovered 9 hours after irradiation of the rabbit with 650 r. on day 6 were well developed, 90 per cent. having primitive streaks, and they showed no sign of abnormal degeneration. The embryos from the animal autopsied 5 hours after exposure to 450 r. on day 6 contained many degeneration granules; there were globules of mucin in all the embryonic discs, 50 per cent. of which were in Stage C and the rest in more advanced stages (Plate 3, fig. P). These few experiments indicate that pre-implantation embryos can be affected by irradiation of the mother. However,

further study would be required to determine in detail the extent of damage and the ability of the embryos to recover from such treatment.

Activity of endometrial carbonic anhydrase

With the exception of the above described results obtained with the specific carbonic anhydrase inhibitor, Neptazane, none of the agents used in this study exerted any inhibitory influence upon the activity of this enzyme in the uterine endometrium.

DISCUSSION

The flat mount preparation was originally devised to provide a simple and rapid method for evaluating the effect upon rabbit blastocysts of culture media, such as might conceivably be used in biochemical *in vitro* experiments. It was, however, envisaged even then that the procedure could be equally useful in studies where experimental factors are made to act upon embryos not directly, but *via* the maternal organism. The present study attests the applicability in this respect of the flat mount preparation. It has enabled us to observe certain morphological features in normal blastocysts and to classify 5- to 7-day normal blastocysts into 6 developmental stages, based mainly on the degree of maturity shown by the embryonic discs. These data, together with the dimensions of the entire blastocysts, served as a basis for assessing the condition of embryos recovered from animals which had been subjected to various treatments. Results obtained with normal blastocysts have brought out the existence of fairly wide limits of morphological variability, within individual members of a litter as well as between different, but chronologically identical, litters. These are findings of some interest and significance: possibly, they explain at least one of the causes underlying the differences in drug susceptibility frequently encountered in the young embryos. Moreover, they indicate that results of histological, chemical, or other analyses, undertaken with single blastocysts, can yield misleading information, and that it is advisable, when studying embryos of polytocous laboratory animals, to use truly representative samples.

We regard the use of animals which have had previous litters as another prerequisite for experiments involving factors likely to exert embryotoxic effects; a fertility record often helps to estimate the effect of a treatment upon the number of embryos.

Of considerable importance to this kind of study is a carefully adjusted schedule as regards the timing of treatment and drug dosage; in most cases these appeared more significant than the route of administration. We recognize that some of the results presented above could be modified by alterations in either timing or dose level: thus, consecutive small amounts of a drug might have induced in blastocysts a condition markedly different from that due to a single large dose.

The substances used for our study belong to categories of agents of which

some could be expected to act predominantly upon the embryo itself, viz. the cytostatic compounds or the metabolic analogues, and of which others were likely to affect primarily the uterine environment, such as the ovarian and other hormones or Neptazane, the sulphonamide inhibitor of endometrial carbonic anhydrase. Some of the embryotoxic materials with which we have treated our experimental animals have also been used by other investigators in studies chiefly concerned with more advanced pregnancy, for the most part in rats or mice. However, we have, in addition, examined the effect of some newer anti-neoplastic drugs not hitherto studied in this respect, namely, Degranol (Vargha, Toldy, Feher, & Lendvai, 1957), and E 39 soluble (Domagk, Petersen, & Gauss, 1954); the former we have found to be relatively non-toxic to the rabbit embryos, but the latter harmful, even in low doses.

The emphasis in this work was on the blastocyst stage, but in several instances observations were also made covering the period from ovulation and fertilization up to and beyond implantation. It was interesting to note that no damage to ovarian function ensued, in so far as ovulation was concerned, in consequence of treatments which proved effective at other stages. This resistance may, of course, be species-specific. On the other hand, fertilization of the ova shed was sometimes affected when large amounts of Thiotepa, TEM, or azaguanine, were administered immediately after mating.

The inclusion in our study of the cleaving egg has permitted us to note how vulnerable the zygote is, during its tubal passage, to the action of agents transmitted from the mother. This fact, amply illustrated by the experiments involving egg transfer and the results obtained with the various cytostatic drugs, disposes of the view occasionally expressed that, because the tubal egg when cultivated *in vitro* shows divisions which fall within physiological time limits, it is largely independent of its environment.

With regard to implantation, when the interval allowed for drug action was brief, as was mostly the case in our experiments, it was seldom possible entirely to prevent blastocyst attachment, even with relatively large doses of potent agents. A rather effective short-term inhibitor of implantation in the rabbit was oestradiol benzoate. In this it differed from diethyl- and dimethylstilboestrol, neither of which prevented nidation, though, like the oestrogenic hormone, both compounds interrupted further foetal development, presumably owing to changes induced in the uterine tissues. Other instances of interference with implantation were the experiments with mercaptopurine, in which a large percentage of blastocysts failed to implant on day 7 if a suitable dose of the purine analogue was injected only some 36 hours earlier. On the other hand, the depression of endometrial carbonic anhydrase activity by Neptazane was ineffective in preventing ovum attachment, perhaps because it proved impossible to achieve complete and lasting inhibition of the uterine enzyme, even with near-toxic doses of this sulphonamide.

Some of the substances which we have administered to pregnant rabbits, for

instance the colchicine derivatives and some alkylating agents, acted equally promptly on all of the investigated stages of pregnancy. But with others, notably the purine analogues mercaptopurine and azaguanine, the effect upon tubal eggs was not discernible, and only became manifest about the time of implantation. This is presumably due to the fact that these antimetabolites must first be incorporated and metabolized to the 'active' derivatives, which become biologically potent and ultimately interfere with embryonic development. Our results suggest that, like tumour and heamopoietic tissue, the early embryo is prominent in its capacity to anabolize purine analogues to such active derivatives. Those few embryos which in spite of exposure survive the treatment, thus appearing resistant to the action of the purine analogues, may be incapable of the 'lethal synthesis', i.e. the conversion of mercaptopurine or azaguanine to the corresponding nucleotides. It would be most interesting to obtain direct proof of this phenomenon experimentally.

However, we have also dealt with metabolic antagonists which, under our experimental conditions, produced relatively little or no effect on rabbit blastocysts. These include the antagonists of folic acid, aminopterin, and amethopterin, and also the analogues of vitamin B_{12}. The vitamin B_{12} analogues, the antagonistic action of which has so far only been demonstrated in certain specialized systems (Cuthbertson, Gregory, O'Sullivan, & Pegler, 1956), obviously lack the ability to interfere with pregnancy in the rabbit, even when administered for extended periods and in large amounts. Although negative, these findings are interesting in view of the fact that the endometrial secretion and blastocyst fluid are exceptionally rich sources of vitamin B_{12} (Jacobson & Lutwak-Mann, 1956), the successful 'replacement' of which by a truly competitive antagonist might have been expected to suppress embryonic development.

Similarly, no adverse effect upon rabbit blastocysts was achieved with excessive doses of vitamin A during the first 5 days of gestation. This contrasts with the well-established adverse effect of hypervitaminosis A in rodents when it occurs in the post-implantation phase (Giroud & Martinet, 1954; Millen & Woollam, 1960).

Another finding worthy of comment was the differential sensitivity within the blastocyst encountered in response to certain agents, as between the embryonic disc and the trophoblast, the former being on the whole more conspicuously affected. We assume that this distinctive susceptibility of the disc and trophoblast, respectively, is the expression of differences in both type and rate of metabolic processes, whereby the more active and rapidly proliferating tissue of the embryo proper suffers adverse effects more readily. Good examples of such differential behaviour were the experiments with Thiotepa, TEM, and E 39 soluble; and also those with mercaptopurine, in which, as late as day $7\frac{3}{4}$, it was possible to recover free-lying, giant vesicles, practically anembryonic yet obviously capable of taking up and retaining water and other constituents characteristic of blastocyst fluid.

On the other hand, with Colcemid, cell-division was affected in both trophoblast and disc within 60 minutes after injection; this was 2–3 hours before the animals developed signs of diarrhoea. In this respect Colcemid differed from Thiolcolciran, another closely related colchicine derivative, which did not induce such striking changes in the rate of cell-division.

The prompt response of the rabbit blastocyst to a variety of extraneous agents, coupled with the straightforwardness of the flat mount preparation, whereby it is equally easy to observe the condition of the embryonic disc and cells within the trophoblast area, combine to suggest a technique suitable for the screening of chemotherapeutic drugs used in malignant diseases, possibly also of cytopathogenic factors such as the viruses. For preference, these should be administered to the pregnant animals in amounts which, in experiments of short duration, do not seriously upset maternal health, but act on the blastocysts either generally or selectively by attacking mainly the embryo proper.

SUMMARY

1. A study was made of the action upon the rabbit embryo of various agents administered to pregnant animals in doses which did not seriously upset maternal health. The pre-implantation blastocyst, examined as a flat mount, was the principal object of study, but observations were also made on ovulation, fertilization, cleavage, nidation, and subsequent phases of pregnancy.

2. To facilitate comparison between embryos from normal and treated animals 5- to 7-day-old blastocysts from untreated animals, grouped according to size, were classified into 6 developmental stages, depending upon the degree of maturity of the embryonic disc. Additional procedures which served to evaluate the condition of blastocysts and their uterine environment were: mitotic counts made in the abembryonic part of the trophoblast, chemical determinations of certain characteristic constituents of blastocyst fluid, and measurement of carbonic anhydrase activity in the endometrium.

3. The experimental agents comprised hormones, colchicine derivatives, several polyfunctional alkylating compounds, metabolic analogues, a sulphonamide inhibitor of carbonic anhydrase, and substances endowed with teratogenic, hepatotoxic, radiation-protective, and hypoglycaemia-inducing properties; total body exposure to X-rays was also investigated.

4. Results obtained at various stages of pregnancy with the different treatments are described and discussed; several results relating to blastocysts are illustrated by photomicrographs.

5. Among significant general findings emerging from the study are: the speed with which exogenous agents are transmitted to embryos even before uterine attachment; differential drug sensitivity, frequently evident in the pre-implantation blastocysts, as between the embryonic disc and trophoblast, the former usually being more susceptible; and variability in response, both individual and between litters, of the embryos to parenterally administered agents.

6. It is suggested that the blastocyst flat mount preparation could be adapted as a conveniently rapid and simple procedure for the screening of cytostatic and other growth-modifying agents.

RÉSUMÉ

L'action des agents divers sur l'embryon de Lapin

1. On a étudié l'action sur l'embryon de Lapin de divers agents administrés à des femelles gravides à des doses qui ne troublent pas sérieusement la santé de la mère. Le blastocyste non encore implanté et examiné par montage *in toto* de sa paroi étalée a été l'objet principal de cette étude, mais des observations ont été également faites sur l'ovulation, la fécondation, le clivage, la nidation et les phases ultérieures de la gravidité.

2. Pour faciliter la comparaison entre les embryons provenant d'animaux normaux ou traités, des blastocystes de 5 à 7 jours provenant d'animaux non traités ont été groupés d'après leur taille et classés en six stades de développement, établis d'après le degré de maturité du disque embryonnaire. D'autres techniques ont été également utilisées pour apprécier l'état des blastocystes et celui du milieu utérin. Ce furent: des numérations de mitoses effectuées sur la partie antiembryonnaire du trophoblaste; des déterminations chimiques de certains constituants caractéristiques du liquide blastocœlien; et des mesures d'activité de l'anhydrase carbonique dans l'endomètre.

3. Les substances expérimentées ont été des hormones, des dérivés de la colchicine, divers composés polyfonctionnels complexants, des analogues métaboliques, un inhibiteur sulfamidé de l'anhydrase carbonique, ainsi que des corps possédant des propriétés soit tératogènes, hépatotoxiques, radioprotectrices, ou produisant de l'hypoglycémie; l'exposition totale du corps aux rayons X a été également comprise parmi les situations examinées.

4. Les résultats obtenus aux divers stades de la gravidité par les différents traitements sont décrits; divers effets se rapportant aux blastocystes sont reproduits dans des illustrations microphotographiques.

5. Parmi les constatations de signification générale qui se sont dégagées de cette étude, on peut citer: la vitesse avec laquelle des agents exogènes sont transmis aux embryons même avant la fixation de ceux-ci à l'utérus; la sensibilité différentielle aux toxiques fréquemment évidente dans les blastocystes non encore implantés, entre le disque embryonnaire et le trophoblaste, le premier étant en général le plus sensible; et aussi la variabilité, à la fois entre individus et entre nichées, quant à la réponse des embryons à des substances administrées par voie parentérale.

6. Il est suggéré que la méthode de préparation des blastocystes étalés par montage *in toto* paraîtrait bien adaptée pour fournir une technique pratique, rapide et simple pour la sélection des substances cytostatiques ou susceptibles de modifier la croissance.

ACKNOWLEDGEMENTS

Several costly substances used in this study were generously donated by pharmaceutical firms: Lederle (Thiotepa, Neptazane), Leda (Degranol), I.C.I. (TEM), Vitamins Ltd., Roche (vitamin A), Ciba (Colcemid), Roussel (Thiolcolciran), Wellcome Foundation (Myleran), Farbenfabriken Bayer AG (E 39 soluble), Organon (desoxycorticosterone acetate). To Dr. E. Lester Smith (Glaxo Laboratories) we are indebted for the gift of vitamin B_{12} analogues, to Sir Charles Dodds for dimethylstilboestrol, and to Professor J. S. Mitchell for help with the irradiation experiments.

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EXPLANATION OF PLATES

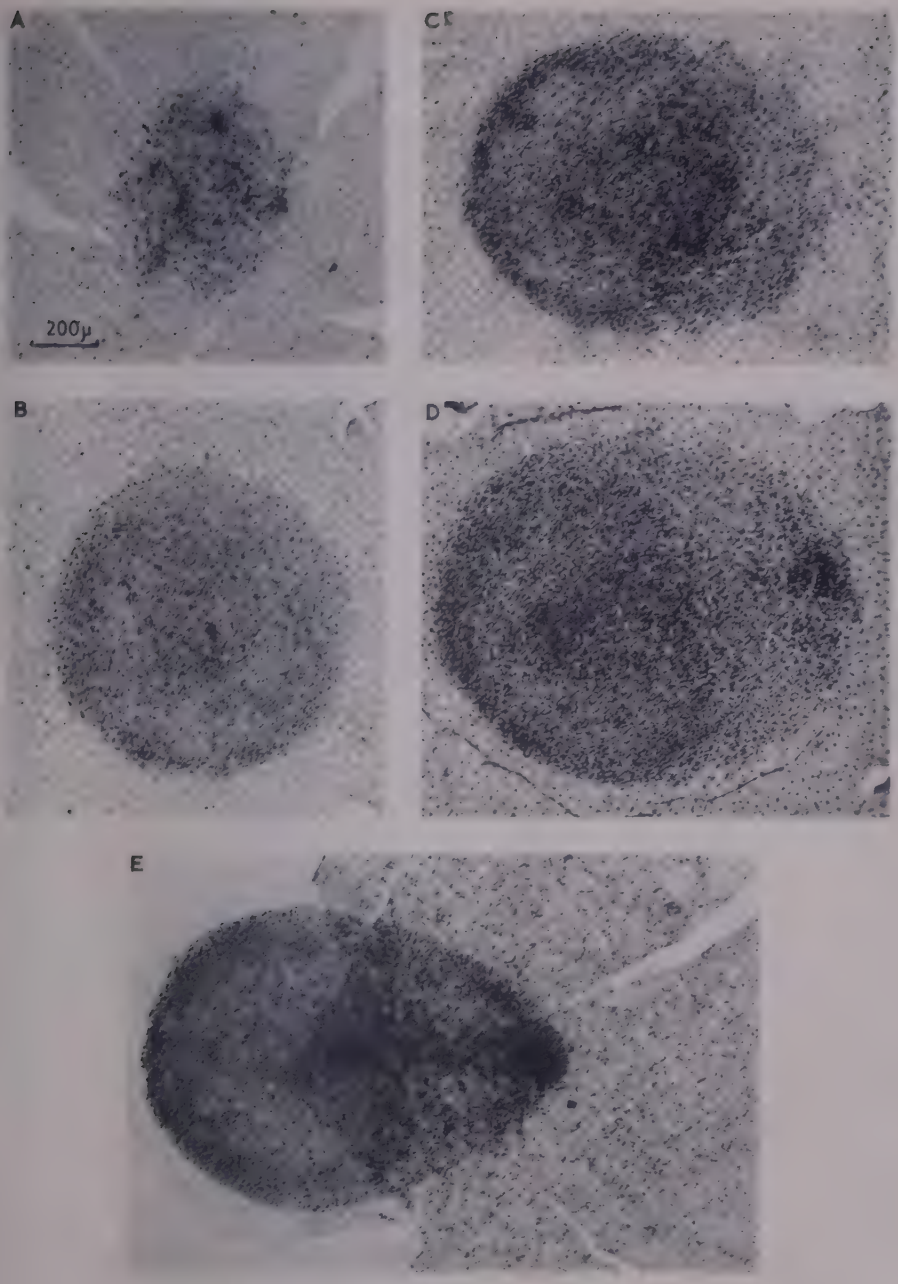
All preparations shown in these plates were stained with Delafield's haematoxylin.

Plate 1

The figures shown in this plate illustrate 5 stages (A, B, C, D, & F) in the normal development of the embryonic disk in 5- to 7-day blastocysts from untreated rabbits. Figs. A–E are all at the same magnification, as indicated in fig. A.

Fig. A. Stage A; 5-day embryo. The disk is 1–2 layers of cells thick and slightly irregular in outline.

Fig. B. Stage B; 6½-day embryo. The disk is about 3 layers of cells thick and has a smooth outline.



C. E. ADAMS, M. F. HAY, *and* C. LUTWAK-MANN

Plate 1

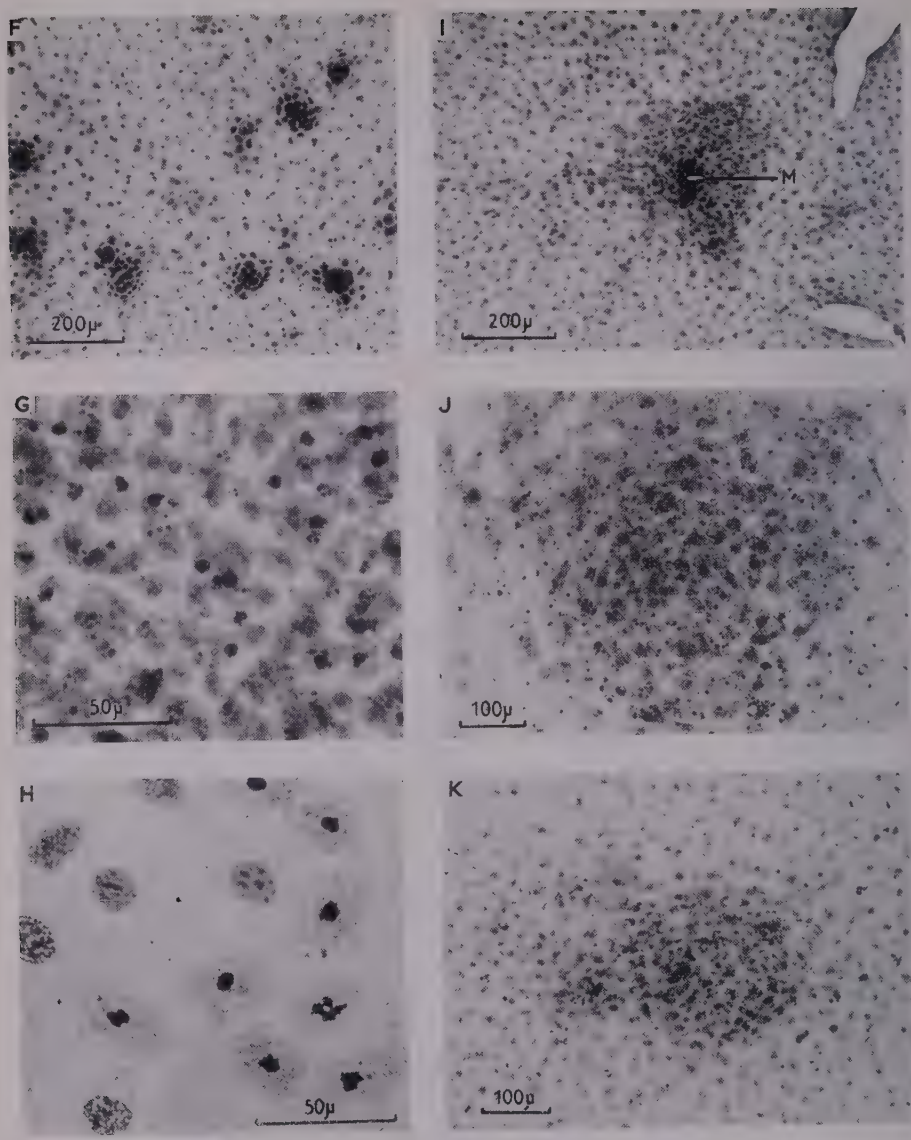


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Plate 2

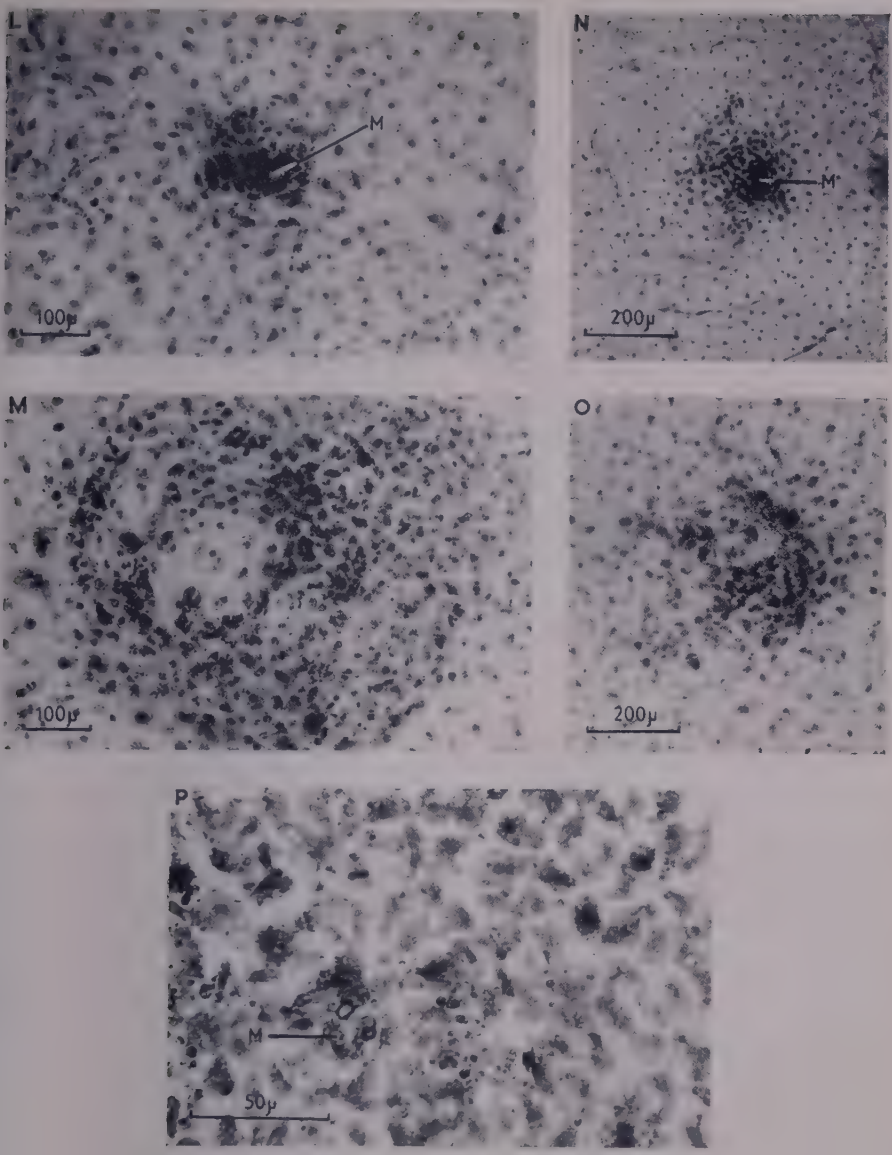

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Plate 3

FIG. C. Stage C; 6-day embryo. The disk has elongated and is beginning to grow out in a posterior direction.

FIG. D. Stage D; 6¾-day embryo. Cells are beginning to condense in the midline of the zone of outgrowth to form the primitive streak.

FIG. E. Stage F; 7-day embryo. The primitive streak is present and stellate mesodermal cells can be seen growing out from the disk in a fan-shaped area round its posterior end. The trophoblast was removed from the anterior end of the disk during preparation.

PLATE 2

FIG. F. Trophoblastic knobs in the equatorial region of the trophoblast of a 7-day embryo. Between the knobs the large nuclei of the trophoderm and the smaller endodermal nuclei can be seen.

FIG. G. Part of the embryonic disk of a 6½-day blastocyst from a rabbit treated 5 hours before autopsy with 2 mg. Colcemid per kg. of body-weight. Cell-division was arrested in metaphase; marked cellular degeneration can be seen.

FIG. H. Part of the abembryonic region of the trophoblast of a 6¾-day blastocyst of a rabbit treated 9 hours previously with 2 mg. Colcemid per kg. body-weight. The trophoblast in this region consists of only one layer of cells and 8 metaphases together with 2 prophases (one shown incompletely) and 2 interphases can be seen; anaphases and telophases were absent from this specimen.

FIG. I. Embryonic disk of a 6½-day blastocyst from a rabbit treated on day 5 with 6 mg. Thiotepa per kg. body-weight. The disk is small and irregular in shape; degeneration granules and a nodule of mucin (M) can be seen. The magnification of this specimen is greater than that of the controls shown in Plate 1.

FIG. J. Embryonic disk of a 6½-day blastocyst from a rabbit treated on day 5 with 2 mg. TEM per kg. body-weight. The disk is only 1–2 layers of cells thick and the cells contain heavily stained granules, probably chromatin; some cells are dividing and an anaphase can be seen close to the edge of the disk. The magnification of this specimen is about twice that of the controls shown in Plate 1.

FIG. K. The embryonic disk of a 6½-day blastocyst from a rabbit that had been treated on day 4 with 0·7 mg. TEM per kg. body-weight. The disk is very small (magnification is the same as in fig. J) and contains numerous degeneration granules. Several mitotic figures can be seen in the trophoblast.

PLATE 3

FIG. L. Very degenerate embryonic disk of a 6¾-day blastocyst from a rabbit treated on day 4 with 2·9 mg. E 39 soluble per kg. body-weight. The disk consists mainly of nodules of mucin (M). Degeneration granules and some dividing cells can be seen in the surrounding trophoblast.

FIG. M. Embryonic disk of a 6½-day blastocyst from a rabbit treated on day 5 with 2·5 mg. E 39 soluble per kg. body-weight. The disk is larger than that shown in fig. L (it is shown here at the same magnification) but it is degenerating.

FIG. N. Embryonic disk of a 6¾-day blastocyst from a rabbit treated on day 1 with 60 mg. 6-mercaptopurine per kg. body-weight. The disk is small and its cells have darkly stained cytoplasm, but there are only a few degeneration granules; a nodule of mucin (M) can be seen.

FIG. O. Embryonic disk of a 6½-day blastocyst from a rabbit treated on days 4 and 5 with 50 mg. 6-mercaptopurine per kg. body-weight. There are more degeneration granules but less mucin than in fig. N. A few mitotic figures can be seen in the surrounding trophoblast.

FIG. P. Part of the embryonic disk of a 6½-day blastocyst from a rabbit that had received 450 r. of X-rays 5 hours before autopsy. Some globules of mucin (M) and severe cellular degeneration are shown.

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Further Investigations on the Cytotoxic and Morphogenetic Effects of Some Nitrogen Mustard Derivatives

by A. JURAND[1]

From the Institute of Animal Genetics, Edinburgh

WITH FIVE PLATES

PREVIOUS experiments with TEM (triethanomelamine) on chick and mouse embryos (Jurand, 1958, 1959) have shown that some embryonic regions are more sensitive than others to its cytotoxic activity. TEM appeared to be specifically active against mesodermal structures, particularly against the somitic mesoderm, being relatively less active against other tissues. In amphibian embryos, however, the neural tube cells were the most sensitive (Waddington, 1958).

N-(*p*-amino-phenyl)-nitrogen mustard (the parent compound) and its acetyl derivative, which have been investigated on chick embryos (Jurand, 1960), have been shown to be specifically toxic to the mesodermal and neural cells. The affected regions were injured primarily at the cellular level, due to the cytotoxic properties of these compounds. The changes at the cellular level were followed by abnormal development of the somites and neural tube.

The present investigations represent further comparative studies on the activity of closely related nitrogen mustard derivatives in chick and mouse embryos, and aim at confirming localized susceptibility to these compounds. In addition to the parent compound and its acetyl derivative, a fluoro-acetyl derivative ('fluorine derivative') was used, viz.

$$FCH_2—CO—NH—C_6H_4—N(CH_2—CH_2—Cl)_2.$$

This compound is considered to be more readily decomposed by hydrolytic enzymes to the parent compound in Walker rat carcinoma cells than is the acetyl derivative and it therefore was expected to be more selective as a carcinostatic factor (Danielli, 1960).

MATERIAL AND METHODS

Experiments on chick embryos were performed with the fluorine derivative only, as the other two compounds had already been examined using this material and the results have been reported (Jurand, 1960).

[1] *Author's address*: Institute of Animal Genetics, West Mains Road, Edinburgh 9, U.K.

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Chick embryos at stage 4 (Hamburger & Hamilton, 1951) were explanted from eggs and cultured *in vitro* according to the method described by New (1955). As the compound is hardly soluble, it was suspended in an appropriate volume of 0·9 per cent. saline solution. One part of this suspension was added to nine parts of liquid albumen, so that the final concentrations of the suspensions were: 10^{-4}, 2×10^{-4}, 4×10^{-4}, and 8×10^{-4} (w/v). The suspensions were administered in amounts of 0·5 ml. round the culture rings used for cultivation of the explanted embryos. Re-incubation took place for 22–24 hours, i.e. until the control embryos had reached 12 or 13 according to Hamburger & Hamilton (1951). In these experiments 109 experimental and 56 control chick embryos were used.

The material was prepared for histological examination by standard methods described previously (Jurand, 1958, 1960). Some of the experimental and control embryos were examined in the electron microscope in order to see the details of the necrotic changes in the neural tube. These embryos were fixed with 1 per cent. buffered osmic tetroxide solution (Palade, 1952), embedded in methacrylate, sectioned with an ultramicrotome, and viewed in a Siemens Elmiscop I.

In order to compare the results obtained from the experiments on chick embryos treated with the fluorine derivative and those reported previously, the specificities of the compounds were examined also on 10-, 13-, and 15-day-old mouse embryos mainly of the JC and to a lesser extent of the JU strain.

Two- to five-months-old females with vaginal plugs were injected subcutaneously under slight anaesthesia with the newly prepared solution or suspension of the drugs. (The parent compound is fairly soluble, but the other two had to be applied in the form of suspensions.) The doses were calculated in mg. per kg. of the actual body-weight as the differences between molecular weights of the compounds concerned are small, and were divided into three equal parts which were injected on three consecutive days, either the 6th, 7th, and 8th days or the 7th, 8th, and 9th days of pregnancy, the day on which the plug was found being regarded as the 1st day. The experimental animals were weighed before each injection and were injected at the same time each day.

The animals were killed by cervical dislocation on the 10th, 13th, or 15th day of pregnancy. After preliminary fixation with Carnoy's fluid (6:3:1) in the intact uterus for about 3 hours, the embryos were dissected and fixed again for another 12 hours in the same fixative. For histological purposes the embryos were dehydrated with absolute alcohol for 6 hours, then cleared in methyl benzoate and embedded in wax. The orientation of the embryos for sectioning was done according to the group of organs to be examined. Sections 6–7 μ thick were stained with methyl green-pyronin. Some embryos were prepared as whole mounts after staining with Mayer's haematoxylin.

For purposes of comparison control mouse embryos of ages from 8 to 15 days' post conception were used. Pregnant control females were anaesthetized on the injection days without being injected.

The preliminary external examination of the embryos after they were excised from the uterus included an assessment of the degree of retardation of development and identification of the external characteristics, such as general body-shape, the line of the vertebral column and spinal cord, the size and shape of the eyes, the presence of liver hernia, the appearance of the limbs or limb-buds, &c. The central nervous system, eyes, mesodermal structures, and, where this seemed necessary, certain other structures were subjected to histological and cytological examination.

The stages of development of the mouse embryos were compared with data available in the literature (Grüneberg, 1943; Snell, 1941) and with the control material.

Table 1 gives the numerical data of the animal material used in all experiments.

TABLE 1

	Number of pregnant females	*Number of embryos*
Parent compound . . .	108	627
Acetyl derivative . . .	160	896
Fluorine derivative . . .	234	1,416
Control	66	528
TOTAL	568	3,467

RESULTS

The effect of the fluorine derivative on chick embryos

The effective dosage of this compound was determined after some preliminary experiments and appeared to be of a range similar to that of the parent compound and its acetyl derivative. Concentrations lower than 2×10^{-4} proved to have very little or no visible influence on development, whereas those higher than 8×10^{-4} were lethal for all experimental embryos. Hence there was a comparatively narrow range of concentrations which could be used for these experiments.

The lowest effective concentration, 2×10^{-4}, did not markedly retard the general development of the embryos. The number of somites averaged not less than the number of somites in the control embryos, but in 19 out of 24 cases the neural tube showed selective reactivity, remaining unclosed either in parts or along its entire length. In the first case the affected parts of the neural tube were the rhombencephalon, the region on the level of the 5th to the 9th somite and the caudal part of the neural plate. The latter remained unclosed so that the sinus rhomboidalis persisted longer than in the control embryos. In embryos of this group the anterior neuropore also failed to close.

In the case of total non-closure of the neural plate or neural groove, which

occurred sometimes after treatment with a concentration of 2×10^{-4} and always after 4×10^{-4}, general development was usually retarded. In cross-sections the neural plate appeared to be open and in most cases completely flat, forming a medullary plate instead of a neural tube. It was flat especially in the head region where it failed to show any depression in the median line (Plate 1, figs. 1, 2). In other cases the medullary plate, unclosed along its entire length, formed rather a V-shaped groove, also in the head region (Plate 1, fig. 3).

When intermediate concentrations were used there was no particular abnormalities in other tissues, except that, because of general under-development, the number of somites was, on average, less than that in control embryos, so that the experimental embryos were found to be only at stage 10 or 11 according to Hamburger & Hamilton (1951). The specificity of the fluorine derivative in these concentrations for the developing neural tube seemed to be more pronounced than in the case of the acetyl derivative.

At the cellular level, in the unclosed medullary plates, frequent abnormalities, confined exclusively to the neural cells, were observed. There were found many giant cells (i.e. hypertrophied cells each with a single nucleus) and cells with pycnotic and necrotically disintegrated nuclei (Plate 1, fig. 4). All these changes occurred alongside normal looking cells (which were in the majority) and those undergoing mitotic division. In some cases, however, mitosis was abnormal, e.g. tripolar metaphase (Plate 1, fig. 5).

After application of still higher concentrations, e.g. 6×10^{-4} and 8×10^{-4}, the neural tissue appeared to be completely destroyed. It consisted entirely of necrotic and deformed cells with very dense, shrunken nuclei staining dark greenish-blue with methyl green. The neural plate was in these cases practically non-existent; its necrotic cells formed only an open, more or less shallow groove (Plate 1, fig. 6). Other tissues of these embryos were also affected, although much less than the neural tube cells. The next most severely affected tissue appeared to be the somitic mesoderm.

Necrotic changes as seen by the electron microscope

The neural tube cells of control embryos at stages 12-13 of development appear in electron micrographs to consist of comparatively large nuclei surrounded by a thin layer of cytoplasm. The electron density of the internal content of the nuclei is slightly different from that of the cytoplasm. In some cases it is less, in others higher, presumably depending on what stage the cell is in its life-cycle. The nucleolus, with a diameter averaging one-fifth to one-quarter of that of nucleus, is much denser than is the nucleus, and contains many small vacuoles. There are also cells with more than one nucleolus and, in some cases, cells with small fragments of nucleolar material alongside the main nucleolus. The nuclear membrane has a double-layered structure. In the cytoplasm there are abundant, often elongated mitochondria with the usual lamellar structure, and yolk granules of all types, as described by Bellairs (1958), although at this stage

they are present in small numbers only, as most have undergone conversion into cytoplasmic constituents (Plate 2, fig. 7).

After treatment with the fluorine derivative there occurred many different necrotic changes which could be distinguished more easily by means of the electron microscope. From histological examination of the experimental embryos it is seen that the first symptom of cytotoxic activity is the enlargement of the cells; in extreme cases they may be referred to as giant cells. These cells may be up to 3 times larger than normal cells. The electron density of all their parts remains similar to that of the control cells. In the cytoplasm, however, signs of necrotic changes can be found, in particular pronounced vacuolization, side by side with large granules which appear to be thick-walled vacuoles, presumably identical with the pyronine-positive granules known from light microscope examination of these embryos. The mitochondria also appear to be enlarged (Plate 2, fig. 8).

Simultaneously with the enlargement of the nuclei, the nucleoli usually also become enlarged in relation to the nuclei, as is shown in Plate 2, fig. 8. In some cases, however, there occurred cells with enlarged nucleoli which showed no distinct change in the size of the nuclei (Plate 2, fig. 8).

Other necrotic changes that follow treatment with higher doses appear mostly in nuclei. The first sign is the dissolution of the nucleolus and the simultaneous gradual vacuolization of the structure of the nucleus which ceases to show its normal fine granular appearance. Underneath the nuclear membrane there appear dense masses which are known to be deeply stainable with nuclear stains. This stage of necrosis with more or less advanced changes in the cytoplasm, is assumed to be karyorrhexis (Plate 2, fig. 10).

In the cytoplasm of these cells further necrotic changes are observed which lead to advanced vacuolar destruction of the cells, resulting eventually in complete disintegration of both nuclei and cytoplasm. Such disintegrated tissue fragments, if examined in the electron microscope, are difficult to recognize as having cellular structure at all. They consist merely of very dense solid masses of homogeneous structureless material which presumably represent remains of nuclei, and complex yolk granules in various, probably abnormal, stages of delayed digestion (Plate 3, fig. 11).

True pycnosis of the nuclei is one of the stages in necrosis. It appears morphologically in various patterns as a less common response to the cytotoxic activity. In such cases the nuclei consist always of denser material, often surrounded by a more homogeneous envelope underneath the nuclear membrane. In general, the nuclei are smaller in these cells, but their cytoplasm is comparatively well preserved (Plate 3, fig. 12).

The effects of the parent compound, acetyl and fluorine derivatives on mouse embryos

Natural prenatal mortality in stages later than the 10th day of gestation

occurs very rarely in the strains of mice used in these experiments. In 34 control females only about 1 per cent. of dead embryos were found on the 15th day of pregnancy. Like TEM, the compounds used in the present experiments cause a high incidence of prenatal mortality if used in doses above a certain threshold. They differ considerably, however, in the level of their threshold dose, and, as in previous experiments on TEM, the embryonic LD_{50} was therefore determined for each of the compounds. The embryonic LD_{50} is the total dose which, if administered subcutaneously in three equal parts on the 7th, 8th, and 9th days of pregnancy, kills by the 15th day of pregnancy about 50 per cent. of implanted embryos. Similarly, the lethal doses (LD_{50}) for adult mice were determined.

Table 2 gives the embryonic LD_{50} as compared with the LD_{50} of the compounds under investigation.

TABLE 2

	LD_{50}	*Embryonic* LD_{50}
Parent compound .	15 mg. per kg.	12 mg. per kg.
Acetyl derivative .	65 ,,	36 ,,
Fluorine derivative .	80 ,,	45 ,,

The parent compound (108 pregnant females). Compared with the other two derivatives, the parent compound shows, in mice, a comparatively small difference between the embryonic LD_{50} and the dose resulting in the prenatal mortality of all embryos, and therefore the doses used in these experiments were restricted to 9, 10·5, 12, and 13·5 mg. per kg. All doses higher than the last named resulted in complete mortality of embryos, or caused the death of the pregnant females 24 hours or more after the last injection. In all cases death was preceded by acute diarrhoea, due to extensive inflammation of the intestinal tract.

After effective doses, injected in three equal parts either on the 6th, 7th, and 8th day or on the 7th, 8th, and 9th day of pregnancy, no selective effects in particular embryonic regions were recorded. The only effect was retardation of development, amounting, on the average, to about 1 day in 10-day-old embryos and up to 3 days in 13-day-old embryos, according to the dose used.

In 36 females injected with the embryonic LD_{50} or with 13·5 mg. per kg. on the 7th, 8th, and 9th day of gestation the 145 surviving embryos showed general retardation of development by 2–3 days. Among these embryos were some with unilateral microphthalmia (12 per cent.) and liver hernia (8 per cent.). Both these abnormalities were macroscopically and histologically of the same kind as those described previously after treatment with TEM (Jurand, 1959).

Microscopical examination of the retarded 10- and 13-day-old foetuses did not reveal any specificity of the parent compound. In less-retarded individuals no tissues appeared to deviate from their normal histological performance, whereas in those more retarded all the tissues were injured to the same extent, showing severe necrotic changes.

Acetyl and fluorine derivatives (394 pregnant females). The ranges of the

effective doses per kg., divided into three equal parts and injected on three consecutive days, were 24–45 mg. for the acetyl derivative and 30–54 mg. for the fluorine derivative. The results will be reported together as there were no qualitative differences between the effects of these two compounds on mice.

In 10-day-old embryos, after injection with low effective doses on the 6th, 7th, and 8th days of gestation, both compounds slowed down the rate of development by 1–2 days in rough proportion to the dose used. The most sensitive region appeared to be the medullary plate which remained open in the majority of embryos of this age group (Plate 4, fig. 13). The rest of the body did not show any abnormalities. After higher doses, however, besides the necrotic changes in the open medullary plate, the mesodermal structures showed scattered necrotic changes (Plate 4, fig. 14).

In 13-day-old embryos the susceptibility of the neural tube tissue was still more pronounced, particularly after injections on the 6th, 7th, and 8th days of gestation. The total doses used in these experiments were 36 or 45 mg. per kg. of the acetyl derivative and 45 or 54 mg. per kg. of the fluorine derivative; in other words, the doses were equal to, or 20–25 per cent. higher than, the respective embryonic LD_{50} of these compounds.

In the surviving embryos, apart from general underdevelopment by up to 3 days, necrotic changes which were confined, after lower doses, almost exclusively to the neural tube tissue, were observed after the use of both compounds. In the majority of surviving embryos of this group the neural tube was found to be closed. This was presumably due to the fact that all the more severely injured embryos with unclosed neural tubes had died before the 13th day of gestation, i.e. before the day of fixation. The extent of necrosis of the neural tube tissue showed a considerable individual variation. In some cases the necrosis was confined to the deeper part of the organ, affecting up to half of it, but in extreme cases the entire cross-section showed necrotic damage (Plate 4, figs. 15, 16, 17, 18).

In cases of confinement of necrosis to the deeper part of the neural tube, although the upper part contained also some randomly scattered necrotically changed cells, such as giant, karyorrhectic, and pycnotic cells, it consisted, in general, of healthy-looking cells, some of which were even in the process of mitotic division. The deeper part of the neural tube of these embryos consisted entirely of necrotic, apparently dead tissue, with deformed nuclei that stained deeply with methyl green. As can be seen in figs. 16, 17, and 18 of Plate 4, there was always present within the lumen of this part of the neural tube a kind of cellular debris which stained mainly red with pyronin. In addition, the damaged part showed in many cases complicated convolutions, whereas the healthy upper part did not deviate in its structure from the axial line (Plate 4, fig. 19).

In this group there were few embryos with necrosis of the deeper part of the neural tube, which was closed as far as the trunk region and open towards its caudal end (Plate 4, fig. 20).

In cases in which the entire neural tube consisted of thoroughly necrotic cells, which occurred particularly after higher doses, extensive changes in some mesodermal structures, e.g. in the somitic mesoderm and mesenchyme cells, were also found. In such embryos the cells in the deepest part of the neural tube had already disappeared, so that in this region the continuity of the neural tube was broken (Plate 4, fig. 18).

A comparison of the effects of the above derivatives on the neural tube after different doses and in different embryos leads to the conclusion that there exists a gradient in the sensitivity of the neural tube to these compounds. The maximum sensitivity is in the deepest part, and it decreases in the opposite direction. This seems to be the reason why the continuity of the neural tube is broken in cases of severe necrosis, as the deepest part, being affected first, undergoes complete necrosis and resorption earlier.

In some cases regeneration of the neural tube presumably took place, so that no, or less severe, necrotic changes were actually present, but the organ showed some structural defects (Plate 4, fig. 21).

In other embryos, after the same doses, the brain and the medullary parts of the neural tube had a typically hydrocephalic appearance. In these cases the widened neural tube, when histologically examined, was seen to have extremely thin walls, in some regions only a single cell thick, showing at the same time scattered necrotic cells (Plate 4, fig. 22).

In a number of embryos of this group, particularly in those with more advanced necrosis of the neural tube, a very distinct difference could be seen between the susceptibilities of the two main components of the developing eye to the cytotoxic activity of the two derivatives. The optic cup was changed necrotically simultaneously with necrotic changes in the deepest part of the neural tube, whereas the lens primordium, which was by this time induced and invaginated, appeared to consist of healthy-looking cells, similar to those in corresponding control embryos (Plate 5, figs. 23, 24).

In the experimental embryos the mesoblast of the limb-buds also showed necrotic changes. In Plate 5, figs. 25 and 26, there are shown dorso-ventral sections through the limb-bud of an experimental embryo and corresponding control. The internal mesoderm of the experimental limb-bud shows extensive necrotic changes, whereas the epidermis and the 2- to 3-celled layer of the adjacent mesoderm are well preserved and do not differ from the corresponding layers of the control limb-bud.

In 15-day-old embryos, which survived the treatment with the embryonic LD_{50}, and in those receiving slightly higher doses (up to 10 per cent. more) injected either on the 6th, 7th, and 8th days or on the 7th, 8th, and 9th days of the gestation, there were found the following morphogenetic malformations: (1) kinking and convolution of the axial organs, (2) unilateral and bilateral underdevelopment of the eye ball resulting in microphthalmia, (3) liver hernia, and (4) shortening of the limbs.

All these anomalies occurred after the use of both acetyl and fluorine derivatives but with higher frequency with the latter. All but the last were identical with those observed after treatment with TEM (Jurand, 1959). Table 3 shows the frequency of the above malformations together with those after the parent compound expressed in percentages:

TABLE 3

*Type of malformation**	*Parent compound*	*Acetyl derivative*	*Fluorine derivative*
Kinking and convolution of the axial organs	—	16	19
Unilateral and bilateral microphthalmia	12	19	22
Liver hernia	8	17	24
Shortening of limbs	—	15	33
Number of embryos investigated	145	242	267

* In many cases embryos in these groups showed more than one of above malformations and therefore were recorded more than once.

DISCUSSION

The present investigations have shown that the two derivatives of aminophenyl nitrogen mustard, unlike the parent compound, first cause abnormalities in the process of neural tube closure if applied in the lowest effective dose at early stages of embryonic development. The appearance of this anomaly is parallel with or is followed by the first, still mild, necrotic changes in the cells of the neural plate, where giant cells and cells in the process of karyorrhexis or pycnosis are randomly scattered between normal cells.

After higher doses the neural plate tissue undergoes more profound necrosis with distinct localization, whereas the remaining structures of the embryo appear to be still normal or show only some minor necrotic changes. In extreme cases, when the dose used is still higher, all embryonic tissues undergo necrosis, resulting in early prenatal mortality and resorption of mouse embryos. Histologically these embryos, if fixed while still alive, resemble embryos treated with the parent compound.

A detailed comparison of all the experimental material of both chick and mouse embryos suggests that the embryonic germ-layers and the embryonic tissues derived from them can be arranged in the following approximate order of decreasing susceptibility to the compounds under investigation: medullary plate or neural tube > mesoderm and mesenchyme > entoderm > ectoderm.

Surviving 15-day-old mouse embryos, if examined histologically, are found to be largely those in which the neural tube injuries have been healed and which show only abnormalities caused by injury to the mesodermal structures similar to that following treatment with TEM (Jurand, 1959). This fact suggests that neural tube injury is much more critical for the future development of the embryo than is injury of the mesodermal structures.

Localized cell necrosis confined to particular embryonic regions has been

reported by many authors working with cytotoxic substances, and with amino-acid and purine analogues. Recently Schultz (1959), for example, reported that the somitic mesoderm of early chick embryos, as well as other tissues, shows extensive pycnotic changes after treatment with Ω-brom-allyl-glycine (BAG) due to its leucine requirement.

The restriction of injury to the neural plate, which remains open and flat both in the chick and in the mouse embryos described in this report, recalls the results of Brachet (1958, 1959) and of Brachet & Delange-Cornil (1959), which were obtained after β-mercaptoethanol treatment of amphibian eggs. This compound was found to inhibit the movements of the presumptive medullary plate cells at the gastrula-neurula stage, which results in a flat medullary plate instead of neural tube without any visible influence on other systems. These authors attribute their findings to the reducing property of β-mercaptoethanol, which maintains the —SH groups in the neural plate cells in the reduced form, thus causing a disturbance in metabolism. It seems reasonable to assume that nitrogen mustard derivatives, after being decomposed by hydrolysing enzymes to their parent compound in tissues containing such enzymes in higher concentration, interfere with the biochemical balance, although they probably do so at a different point in the metabolic process. It is widely accepted that the alkylating agents disturb the mechanism of DNA synthesis (Bodenstein, 1954) and as a result they disturb the synthesis of specific proteins (Danielli, 1954) indispensable for the morphogenetic development of the sensitive organ.

It is true that the spontaneous failure of neural tube closure called platyneury occurs sometimes in normal chick embryos but this phenomenon is always associated with anomalies in somite formation (Grünwald, 1935). In the present investigations no such anomalies in the somites of experimental embryos with neural plate non-closure were found after fluorine derivative, and the frequency of the injury was so high, even when the lowest effective concentration was used, that this effect cannot be compared with spontaneous platyneury. Moreover, there were no control embryos similar to the experimental ones. Last, but not least, spontaneous platyneury in mouse embryos is not known and yet the acetyl and fluorine derivatives, if used in proper dosage, prevent the closure of the neural tube.

In addition to causing injury to the medullary plate, both the acetyl derivative, as reported previously (Jurand, 1960), and the fluorine compound (particularly in higher dosage) have an adverse effect on the mesoderm. In the present investigations the fluorine derivative caused necrosis of the mesoblast in the limb-buds, leaving unharmed the outer layer, which is 2–3 cells thick, and the limb-bud epidermis, as well as the apical ectodermal ridge. This fact suggests that the epidermis and the underlying thin mesoblast layer are less susceptible than the deeper mesodermal cells. What causes the difference between the outermost and the internal cells of the mesoblast is not known. Possibly there are some biochemical similarities between the external layer of the mesoblast and the

epidermal cells due to their close proximity anatomically, but it may also be that the outermost layer of the mesoblast has some relation to the so-called 'refractile' layer of the mesoblast, which seems to play an epidermis-like role in limb differentiation. This problem has recently been the subject of discussion (Bell, Saunders, & Zwilling, 1959; Grüneberg, 1960).

As far as the process of necrobiosis is concerned, it is well known that it is basically a natural process occurring in practically all living tissues, both in those in the course of differentiation and growth and those of adult organisms. The different stages of necrosis resulting from the cytotoxic activity of the compounds under investigation seem to be of the same nature as those described by many authors in normal tissues, like those in the regressing tissues of tadpoles' tails, or developing tissues (Leuchtenberger, 1950; Glücksmann, 1951; and others). It seems probable that cell death, regardless of its cause, follows the same pathway and results in the same cytological abnormalities of the nucleus, the cytoplasm, or both.

When the present data are looked at from a more general point of view some confirmation can be found of certain principles of teratogeny put forward by Wilson (1960). His second principle states that in some cases different agents produce characteristic patterns of defects due to similar action upon specific phases of metabolism in embryonic tissues. In other words, it seems that these characteristic patterns may result from the similar susceptibility of certain embryonic tissues to different agents, e.g. TEM, acetyl and fluorine derivatives, in respect of their activity against mesodermal structures. This might be the reason that in 15-day-old mouse embryos the teratological effects following the use of compounds under investigation are similar to those after triethanomelamine.

In conclusion, it should be emphasized that the influence of the acetyl and fluorine derivatives may be regarded as specific with regard to their morphogenetic effect on the neural plate non-closure; but the necrotic changes in the attacked organs are in general the same as after other radiomimetic agents. Some of the affected cells break down soon after treatment and others, arrested at the interphase, continue to grow and to reach giant proportions.

SUMMARY

The cytotoxic and morphogenetic activity of N-(*p*-amino-phenyl)-2, 2′-dichlorodiethylamine and its acetyle and fluoro-acetyl derivatives was studied on chick and mouse embryos.

In chick embryos the fluorine derivative showed a specific affinity for the medullary plate, causing inhibition of neural tube closure at lower concentrations and complete destruction of the medullary plate at higher concentrations.

Electron microscope observations of the affected neural cells showed some examples of necrotic changes after treatment with the fluorine derivative in chick embryos.

The parent compound and its acetyl and fluorine derivatives slow down the developmental rate of the mouse embryos. Here also, the tissue most sensitive to acetyl and fluorine derivatives appeared to be the neural tube, particularly it deepest part. The extent to which it is affected depends on the dose of the cytotoxic agent, so that a gradient in the sensitivity of the neural tube seems likely.

After higher doses the cytotoxic influence extends to other embryonic tissues which can be arranged as follows according to decreasing sensitivity: medullary plate or neural tube > mesoderm and mesenchyme > entoderm > ectoderm.

Older (i.e. 15-day-old) mouse embryos show malformations due to injury to the mesodermal structures, presumably because those with neural tube injuries either fail to survive or regenerate them. In the last case the injury to the mesodermal structures result in anomalies like convolution and kinking of axial organs, liver hernia, microphthalmia, &c.

RÉSUMÉ

Nouvelles recherches sur les effets cytotoxiques et morphogénétiques de quelques dérivés de l'ypérite nitrée

On a étudié, sur des embryons de poulet et de souris, l'activité cytotoxique et morphogénétique de la N-(*p*-amino-phényl)-2,2′-dichlorodiéthylamine et de ses dérivés acétylé et fluoro-acétylé.

Sur l'embryon de poulet, le dérivé fluoré a montré une affinité spécifique pour la plaque médullaire, inhibant la fermeture du tube nerveux aux concentrations les plus faibles, et détruisant complètement la plaque médullaire aux concentrations élevées.

Observées au microscope électronique, les cellules neurales atteintes montraient quelques exemples de modifications nécrotiques après un traitement par le dérivé fluoré, chez l'embryon de poulet.

Le composé initial et ses dérivés fluoré et acétylé ralentissent le rythme de développement des embryons de souris. Ici encore, le tube nerveux apparaît comme étant le tissu le plus sensible aux dérivés fluoré et acétylé et, en particulier, dans sa région la plus profonde. L'extension des lésions dépend de la dose du facteur cytotoxique, de sorte que l'existence d'un gradient de sensibilité du tube nerveux paraît vraisemblable.

Aux doses élevées, l'influence cytotoxique s'étend aux autres tissus embryonnaires, qui peuvent être rangés comme suit selon leur sensibilité décroissante: plaque médullaire ou tube nerveux > mésoderme et mésenchyme > endoderme > ectoderme.

Les embryons de souris plus âgés (15e jour) présentent des malformations dues aux lésions des structures mésodermiques, sans doute parce que ceux dont le tube nerveux a subi des lésions ou bien ne survivent pas, ou bien les réparent. Dans ce dernier cas, les lésions des structures mésodermiques provoquent des

anomalies telles que: enroulement et plissement des organes axiaux, hernie hépatique, microphtalmie, &c.

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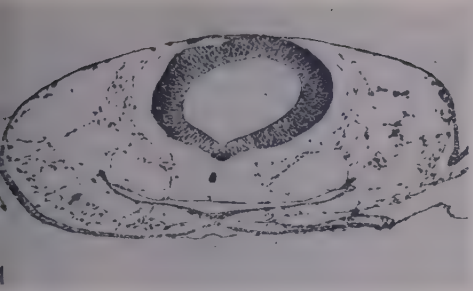

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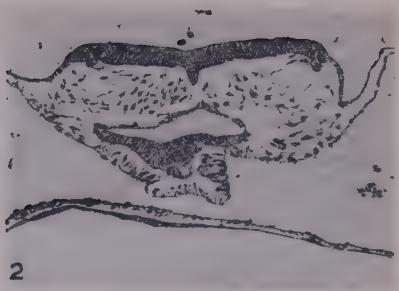

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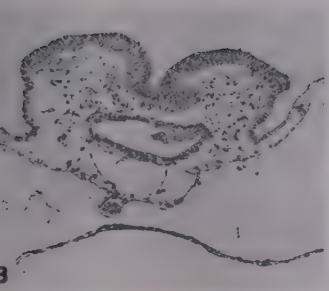

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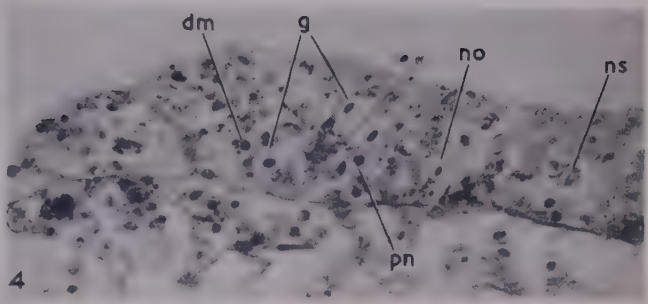

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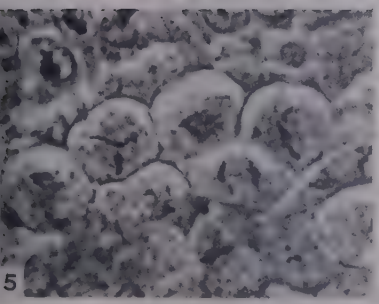

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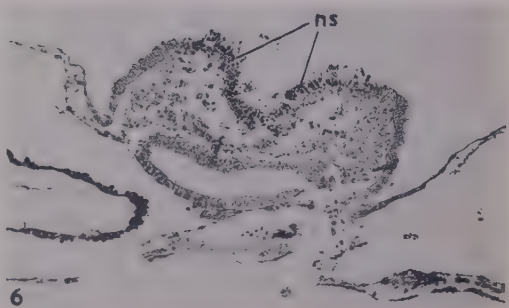

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A. JURAND

Plate 1

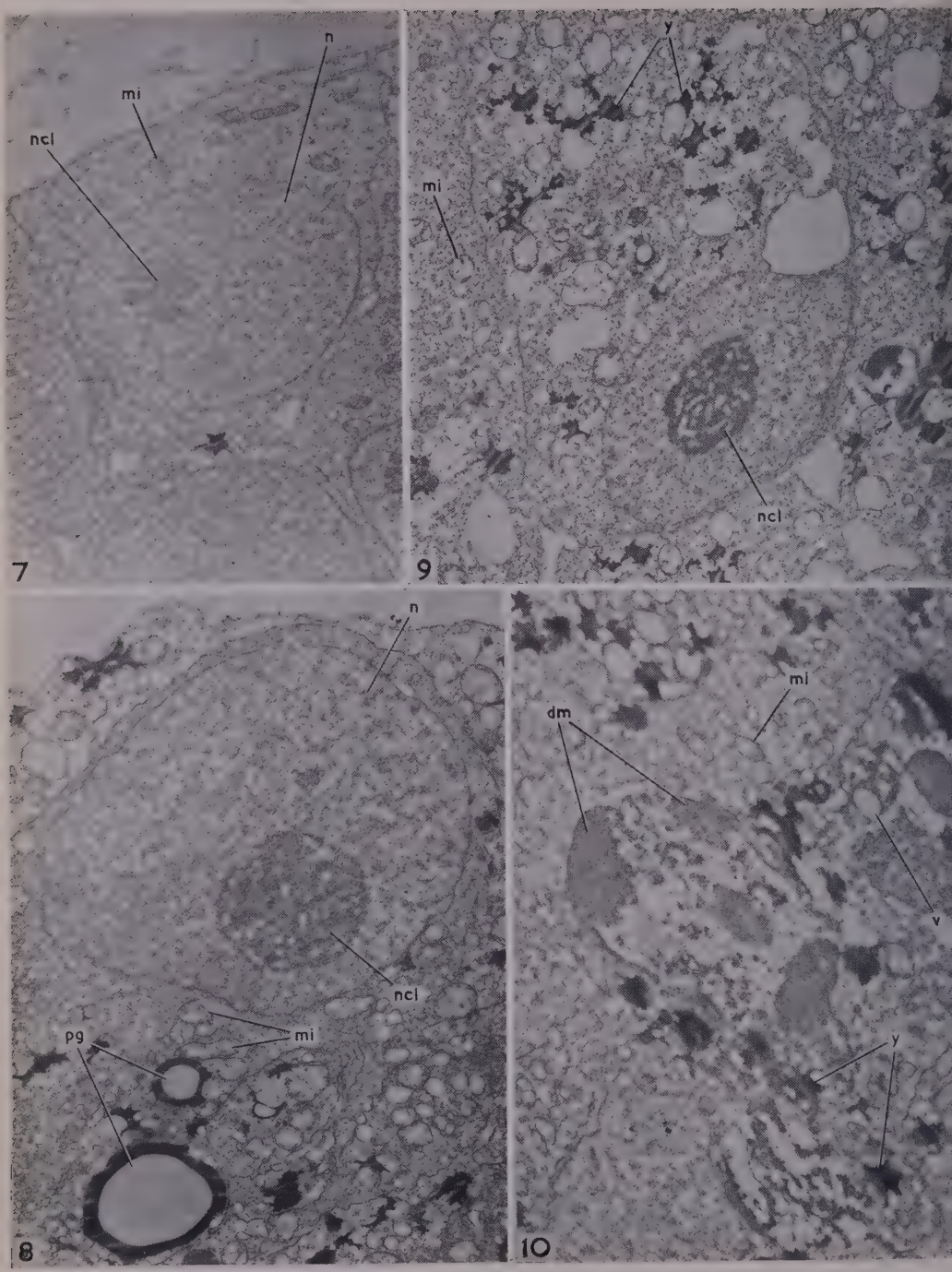

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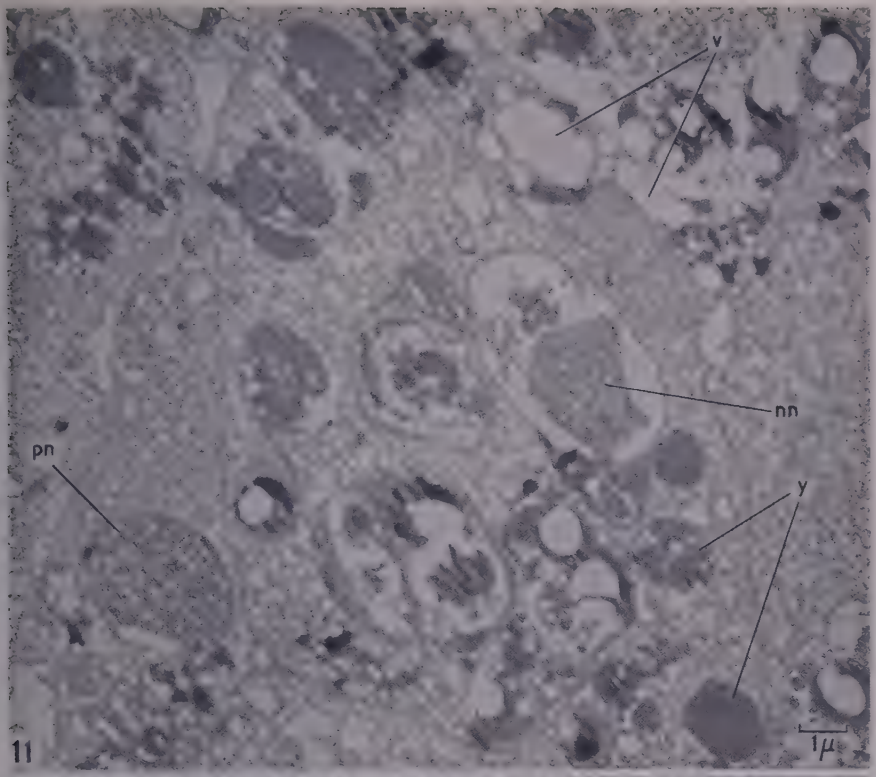
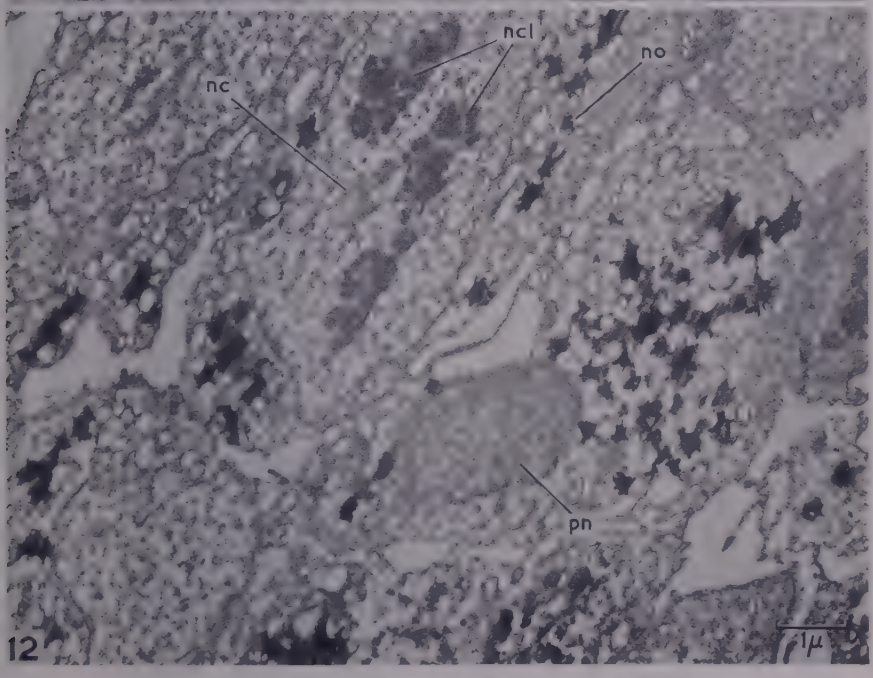
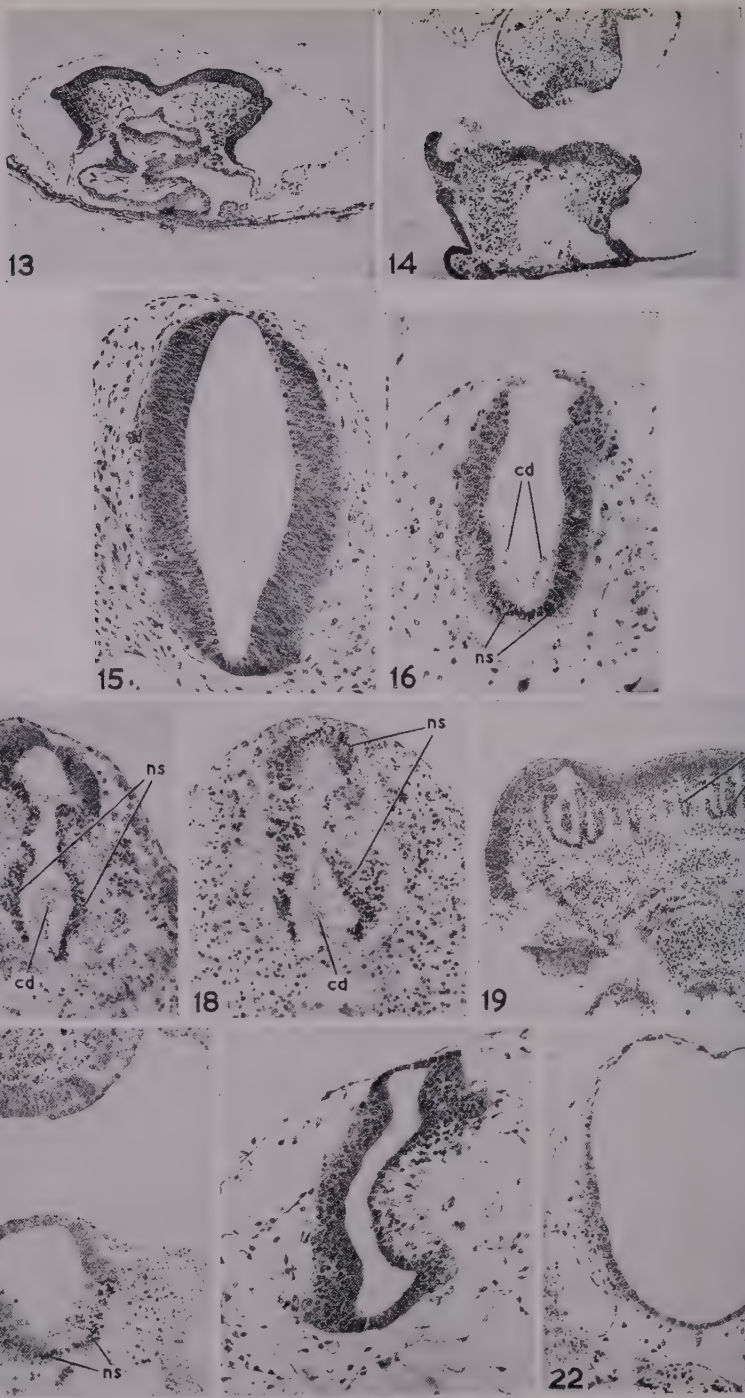
Plate 2





A. JURAND

Plate 3



A. JURAND

Plate 4

EXPLANATION OF PLATES

Abbreviations: *cd*, cellular debris; *dm*, dense masses of necrotic chromatin; *ec*, eye cup; *g*, giant cells; *lp*, lens primordium; *mi*, mitochondria; *nc*, nucleus; *ncl*, nucleolus; *nn*, nuclear necrosis; *no*, normal cell; *ns*, necrosis; *pg*, pyronin positive granules; *pn*, pycnotic nucleus; *v*, vacuolization of cytoplasm; *y*, yolk granules.

Plate 1

Fig. 1. Transverse section through the head of a control chick embryo (stage 12). ×80.

Fig. 2. Transverse section through the head of a chick embryo treated with fluorine derivative in concentration 2×10^{-4}. Note the flat neural plate. ×80.

Fig. 3. Transverse section through the head of a chick embryo treated with fluorine derivative (4×10^{-4}). Note a few necrotic cells at the bottom of the groove. ×80.

Fig. 4. Portion of an open neural plate of a chick embryo treated with fluorine derivative (4×10^{-4}). ×420.

Fig. 5. Tripolar metaphase in the neural plate of a chick after treatment with fluorine derivative. ×700.

Fig. 6. Transverse section through the head of a chick embryo treated with fluorine derivative (8×10^{-4}). Note complete necrosis of neural cells. ×80.

Plate 2

Fig. 7. Electron micrograph of a control neural tube cell (chick). *mi*, mitochondria; *nc*, nucleus; *ncl*, nucleolus. ×8750.

Fig. 8. Electron micrograph of a giant cell in the chick neural plate after treatment with fluorine derivative (4×10^{-4}). ×8750.

Fig. 9. A chick neural tube cell with an enlarged nucleolus (*ncl*)–fluorine derivative (4×10^{-4}). ×8750.

Fig. 10. A chick neural tube cell in the stage of karyorrhexis after treatment with fluorine derivative (6×10^{-4}). Note the dense masses of necrotic chromatin (*dm*) at the periphery of the nucleus. ×8750.

Plate 3

Fig. 11. Severely damaged neural tube tissue after treatment with fluorine derivative (8×10^{-4}). Note an advanced stage of nuclear necrosis (*nn*) in comparison with slightly less injured pycnotic nucleus (*pn*) in the lower left side of the photograph and yolk granules (*y*) in different stages of preservation. ×6600.

Fig. 12. Neural tube cells in chick after treatment with fluorine derivative (6×10^{-4}). In the middle there is a cell with a dense pycnotic nucleus (*pn*), beside a normal cell (*no*) with well-preserved nucleoli (*ncl*). ×10000.

Plate 4

Fig. 13. Transverse section through the head of a 10-day-old mouse embryo after treatment with fluorine derivative (total dose 36 mg. per kg.). ×60.

Fig. 14. Transverse section through the head of a 10-day-old mouse embryo after treatment with acetyl derivative (total dose 45 mg. per kg.) ×60.

Fig. 15. Transverse section through the neural tube of a control 13-day-old mouse embryo. ×115.

Fig. 16. Transverse section through the neural tube of a 13-day-old mouse embryo treated with fluorine derivative (total dose 36 mg. per kg.). Note the necrotic changes (*ns*) at the bottom of the neural tube and the cellular debris (*cd*). ×115.

Fig. 17. Transverse section through the neural tube of a 13-day-old mouse embryo treated with fluorine derivative (total dose 42 mg. per kg.). Necrotic changes (*ns*) occupy more than half of the neural tube. ×115.

Fig. 18. Similar section after treatment with acetyl derivative (total dose 45 mg. per kg.). Note extensive necrosis (*ns*) in all tissues. ×115.

Fig. 19. Longitudinal section through a 13-day-old embryo treated with fluorine derivative (total dose 45 mg. per kg.). Note the comparatively well-preserved upper part of the neural tube and necrosis (*ns*) of the lower part, with convolutions. ×60.

Fig. 20. Transverse section through a 13-day-old mouse embryo after treatment with acetyl derivative (total dose 42 mg. per kg.). Note partial necrosis (*ns*) of the deeper part of the neural tube in the

trunk region and the open neural plate in the tail region. Retardation in the development amounted in this case to about 3 days. ×100.

FIG. 21. Transverse section through the neural tube which regenerated after injury by fluorine derivative (total dose 36 mg. per kg.). ×115.

FIG. 22. Transverse section through the hydrocephalic neural tube after treatment with fluorine derivative (total dose 42 mg. per kg.).

PLATE 5

FIG. 23. Section through the developing eye of a control 11-day-old mouse embryo. ×330.

FIG. 24. Section through the developing eye of a 13-day-old mouse embryo treated with fluorine derivative (total dose 42 mg. per kg.). Note well-preserved lens primordium (*lp*) and many necrotic changes (*ns*) in the eye-cup. ×330.

FIG. 25. Dorso-ventral section through the limb-bud of an 11-day-old control mouse embryo. ×125.

FIG. 26. Dorso-ventral section through the limb-bud of a mouse embryo treated with fluorine derivative (total dose 48 mg. per kg.). Note the well-preserved epidermis and the adjacent layer of the mesoderm in comparison with necrosis (*ns*) of the internal part of the mesoblast. ×125.

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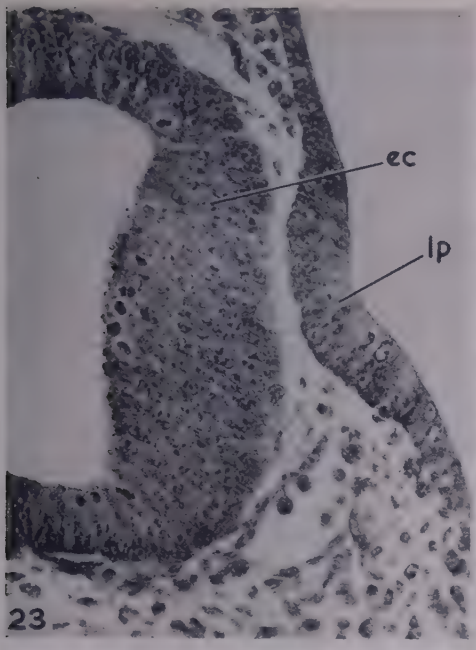

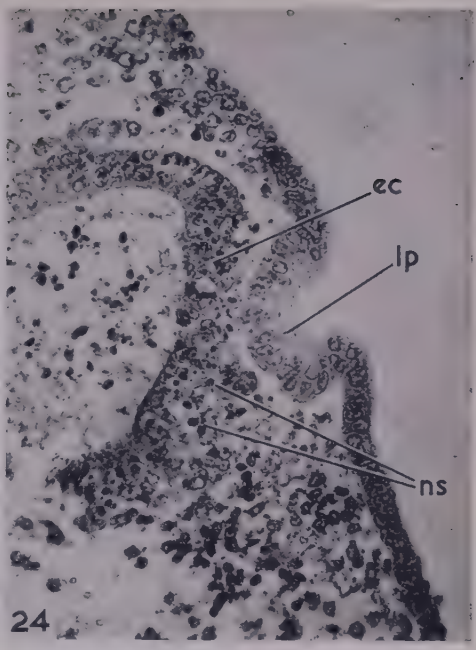

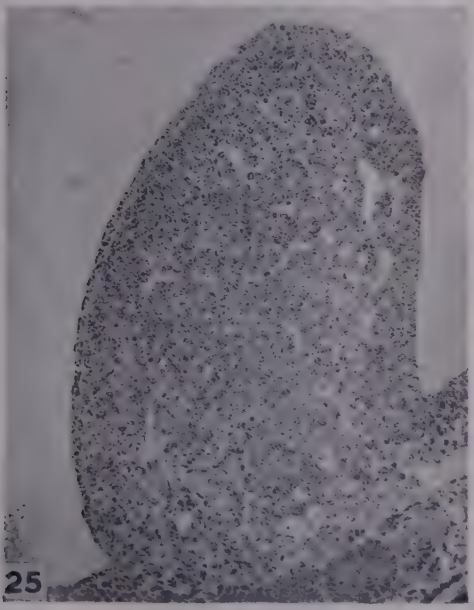

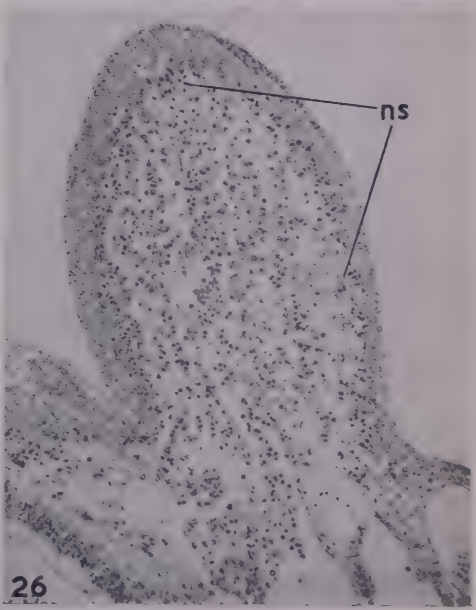

A. JURAND

Plate 5

Investigations of the Germ-plasm in Relation to Nuclear Transplantation

by MARIE A. DI BERARDINO[1]

From the Institute for Cancer Research, Philadelphia

WITH PLATE

INTRODUCTION

THE origin of the germ-cells has been extensively investigated in both invertebrates and vertebrates. In invertebrates it has been traced to the early cleavage of the zygote, e.g. in such forms as *Ascaris megalocephala* (Boveri, 1887) and *Sciara* (Metz, 1938). Within the vertebrate group the cases in which primordial germ-cells have been detected in cleavage stages are very few. Eigenmann (1891) identified gonocytes of *Micrometrus aggregatus* in the late gastrula stage on the basis of their large size and the uniform distribution of their chromatin. The first clear demonstration of vertebrate primordial germ-cells appearing in a stage as early as the blastula was made in the European frog, *Rana temporaria* (Bounoure, 1934). In this case germ-cells were found to be conspicuous because of a stainable cytoplasmic element, the germ-plasm. This germ-plasm first appears shortly after fertilization in the form of islets concentrated in the vegetal pole region of the egg (Bounoure, 1934, 1939, 1954). In these investigations Bounoure traced the plasm-bearing cells to the blastula stage, at which time the germ-plasm is incorporated into cells near or on the floor of the blastocoele. In later stages of embryonic development these primordial germ-cells were traced to the genital folds. That these cells are directly ancestral to the mature germ-cells was indicated by subjecting the vegetal pole of *R. temporaria* eggs to ultraviolet irradiation shortly after fertilization (Bounoure, 1939, 1954). Eggs treated in this way developed into metamorphosed animals whose gonads, reduced in size, possessed only a small quantity of germ-cells, and in a few cases were devoid of germ-cells. More recently, Blackler (1958) has confirmed Bounoure's studies in *R. temporaria* and has also made comparable studies on other Anuran species. In *Bufo bufo* and *Xenopus laevis*, the presence of germinal cytoplasm corresponded, in general, to that of *R. temporaria*; however, no germinal cytoplasm was seen in gastrulae or neurulae of *R. esculenta*. Grafting experiments (Blackler, 1960) have demonstrated that the 'germ-cell region' of *X. laevis*

[1] *Author's address*: Institute for Cancer Research, 7701 Burholme Avenue, Fox Chase, Philadelphia 11, Pa., U.S.A.

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neurulae is directly ancestral to the mature gametes. When the 'germ-cell region' of a two-nucleoli *Xenopus* neurula was replaced with the 'germ-cell region' of a neurula whose cells contain only one nucleolus, the resultant mature animal produced germ-cells with only one nucleolus, and when the latter was mated with a normal 2 N frog, it yielded in the F_2 generation a Mendelian ratio of 1-nucleolar frogs.

Comparable information on the localization of primordial germ-cells in the early developmental stages of *R. pipiens* has not been available. Since this species is being used extensively in nuclear transplantation studies of blastula and early gastrula stages (King, Briggs, & Di Berardino, 1959; Briggs & King, 1960), it has become necessary to know whether or not germ-cells can be distinguished from somatic cells and, if so, what their numbers and localizations are. The present communication presents cytological studies on primordial germ-cells in the mid-blastulae and early gastrulae of *R. pipiens*.

MATERIALS AND METHODS

R. pipiens blastulae, stage 8, and early gastrulae, stage 10 (Shumway, 1940), were selected for study because these stages corresponded to the stages from which donor nuclei were taken for nuclear transplantation studies. The eggs were fixed and stained according to the techniques of Bounoure (1934) and Blackler (1958). In general, the procedure consisted of a 5-minute hot neutral formalin fixation followed by soaking in warm, saturated potassium dichromate for 42 hours. Eggs were sectioned at 5μ, stained with the Altmann acid fuchsin mitochondrial stain and counterstained with aqueous Azure A. Additional eggs were handled with slight modifications; namely, some eggs were fixed in Smith's, some were sectioned at 10μ, and in some cases acid fuchsin staining was omitted. The use of these modifications simplifies the histological technique and gives a satisfactory demonstration of the germ-plasm.

RESULTS

The Altmann mitochondrial stain, acid fuchsin, stains the ground cytoplasm, yolk platelets, and mitochondria red; the counterstain, Azure A, colours the germ-plasm purple. The affinity of the germ-plasm for Azure A would be expected, since Azure A is a good stain for revealing nucleic acids, and Blackler (1958) established that the germ-plasm contains RNA. The germ-plasm exists in the form of a distinct purple islet(s) in the cytoplasm and, depending on the stage of development, may be situated near the cell membrane or close to the nucleus (Plate, figs. A, C). Occasionally, the germ-plasm may be seen in the form of a ring (Plate, figs A, B).

Stage 8

All of the thirteen blastulae studied contained germ-plasm. The number of cells possessing the germinal cytoplasm varies among the individual blastulae;

this range extends from 5 to 14 cells (Table 1). The primordial germ-cells are situated in the vegetal hemisphere, and at this stage 84 per cent. are found in the upper third. This area corresponds to the floor of the blastocoele and approximately the second and third layers subjacent to the blastocoele cavity. The germ-plasm is usually situated near the cell membrane (54 per cent. of the cells) or it may be near the nucleus; however, it is rarely seen at this stage adjoining the nucleus (Table 1).

TABLE 1

Germ-plasm in Rana pipiens (*stage 8*)

Egg	*Number of cells containing germ-plasm*	*Location in vegetal hemisphere*			*Location in cell*			
		Upper third	*Middle third*	*Lower third*	*Near cell membrane*	*Half-way between cell membrane and nucleus*	*Near nucleus*	*Adjoining nucleus*
1	5	—	3	2	5	—	—	—
2	5	3	2	—	1	2	2	—
3	6	6	—	—	4	2	—	—
4	7	6	1	—	2	3	1	1
5	8	8	—	—	—	—	8	—
6	9	9	—	—	5	1	3	—
7	9	9	—	—	4	1	4	—
8	9	6	3	—	3	3	3	—
9	9	8	1	—	2	4	3	—
10	10	8	—	2	9	1	—	—
11	11	10	1	—	8	3	—	—
12	12	9	1	2	7	5	—	—
13	14	14	—	—	11	2	—	1
Total no.	114	96	12	6	61	27	24	2
Percentage	100	84·1	10·5	5·3	53·5	23·7	21·0	1·8

Stage 10

Eleven out of twelve gastrulae contained cells with germ-plasm, and the number of cells displaying the plasm ranges from 4 to 15 cells (Table 2). As in stage 8 the majority of stage-10 cells containing germ-plasm is also present in the upper third of the vegetal hemisphere; however, the percentage is lower (64 per cent. for stage 10 compared with 84 per cent. for stage 8). Table 2 illustrates that a slight shift of plasm-bearing cells to the middle-third of the vegetal hemisphere has occurred in the early gastrula. The most striking difference between stage 8 and stage 10 plasm-bearing cells is in the intracellular location of the germ-plasm. At stage 10, 84 per cent. of the primordial germ-cells contain the germ-plasm in a juxtanuclear position (Table 2). In stage 8 the germ-plasm is situated near the cell membrane (53·5 per cent. of cells) or at intermediate locations (44·7 per cent.), and is only rarely found directly surrounding the nucleus (1·8 per cent.).

TABLE 2

Germ-plasm in Rana pipiens (*stage 10*)

Egg	Number of cells containing germ-plasm	Location in vegetal hemisphere			Location in cell			
		Upper third	Middle third	Lower third	Near cell membrane	Half-way between cell membrane and nucleus	Near nucleus	Adjoining nucleus
1	0	—	—	—	—	—	—	—
2	4	4	—	—	—	—	—	4
3	5	—	5	—	—	—	—	5
4	6	1	5	—	—	—	—	6
5	7	6	1	—	—	—	—	7
6	9	1	7	1	1	2	5	1
7	9	7	2	—	1	—	2	6
8	11	9	2	—	—	—	—	11
9	11	7	3	1	—	—	—	11
10	12	9	2	1	2	2	1	7
11	14	11	3	—	—	1	—	13
12	15	11	4	—	—	—	—	15
Total no.	103	66	34	3	4	5	8	86
Percentage	100	64·0	33·0	2·9	3·9	4·9	7·7	83·5

DISCUSSION

The present study of germ-plasm in *R. pipiens* blastulae and gastrulae closely parallels that reported by Blackler (1958) for comparable stages in *R. temporaria*, *B. bufo*, and *X. laevis*. In *R. pipiens* mid-blastulae and early gastrulae a limited number of cells (from 4 to 15) contain a distinctive cytoplasmic component, the germ-plasm, which stains purple after Azure A staining. All of these cells are confined to the presumptive endoderm. Within these cells the germ-plasm occupies a more or less peripheral position at the blastula stage, then at the beginning of gastrulation the germ-plasm surrounds the nucleus.

Since the primordial germ-cells of *R. pipiens* can be localized *in situ*, some correlation can be made with the *in vivo* nuclear transplantation studies. King *et al.* (1959) and Briggs & King (1960) tested nuclei from different areas of the animal and vegetable hemispheres of the early gastrula as well as animal hemisphere nuclei from the mid-blastula. Since no primordial germ-cells are present in the animal hemisphere, and very small numbers of gonocytes are present in the vegetable hemisphere, these nuclear transplantation studies as well as previous ones involving these stages of *R. pipiens* (see Briggs & King, 1959, for references to the earlier work) must have involved entirely or almost entirely somatic nuclei.

What appears most significant is that somatic nuclei can promote the formation not only of somatic cells, but also germ-cells. Briggs & King (1960) found that somatic cell nuclei, which still retain the capacity to promote the formation

of the normal array of somatic cells, also retain the capacity to promote germ-cell formation. However, among larvae derived from blastula animal hemisphere nuclei, they found that fewer than expected possessed ovaries with growing oocytes. The interpretation of this result remains uncertain until a microscopic analysis of the gonads is performed. Nevertheless, some somatic nuclei appear to promote germ-cell formation. Similarly, Gurdon *et al.* (1958) have obtained sexually mature individuals from transplantation of *Xenopus* endoderm nuclei; these endoderm donor nuclei are derived from areas not containing primordial germ-cells. The explanation in the cases of *R. pipiens* and *X. laevis* may be that, since the germinal cytoplasm is a normal constituent of the unfertilized egg, activation of the host egg by the transplantation technique may be sufficient to 'organize' the germinal cytoplasm in the unfertilized egg. Blackler (1958) has shown that *R. temporaria* parthenogenetic blastulae contain cells with germ-plasm, and he has suggested that the appearance of germ-plasm in the recently fertilized egg may be part of the cortical reaction that follows fertilization.

The question arises whether germ-cell nuclei can promote the formation not only of germ-cells, but also of somatic cells. Such a test requires definite identification of living germ-cells in various stages of development. Preliminary attempts to identify living gonocytes by means of vital staining were made by Blackler (1959). Vital staining carried out by us has revealed that neutral red 'selectively' stains a few cells on the floor of the blastocoele. Whether or not these stained cells do in fact contain germ-plasm has not yet been determined.

SUMMARY

1. Primordial germ-cells in *R. pipiens* mid-blastula and early gastrula stages were detected in serial sections, because these cells contained a stainable component, the germ-plasm.

2. Their location was of prime interest in determining whether or not the nuclei of primordial germ-cells had been used in previous transplantation studies (King *et al.*, 1959; Briggs & King, 1960).

3. In the mid-blastula and early gastrula stages the primordial germ-cells were confined to the vegetal hemisphere. The majority of these cells was situated in the upper-third of the vegetal hemisphere.

4. An intracellular displacement of the germ-plasm occurred between mid-blastula and early gastrula stages.

5. The number and location of the primordial germ-cells suggest that only in rare cases could a nucleus from one of these cells have been transplanted in the previous studies, and such cases would probably have been undetectable. What appears most significant is that nuclei derived from cells not containing germ-plasm are capable of giving rise to larvae whose gonads contain numerous auxocytes.

RÉSUMÉ

Recherches sur le plasme germinal, en liaison avec les expériences de transplantation nucléaire

1. On a découvert les cellules germinales de *Rana pipiens* sur les coupes sériées des stades de la blastula moyenne et de la jeune gastrula, grâce au fait que ces cellules renferment un composant colorable, le plasme germinal.

2. Leur localisation présentait un grand intérêt pour déterminer si les noyaux des gonocytes primordiaux ont été ou non utilisés dans des expériences antérieures de transplantation (King *et al.*, 1959; Briggs & King, 1960).

3. Dans la blastula moyenne et la jeune gastrula, les gonocytes primordiaux sont confinés à l'hémisphère végétatif. La majorité d'entre eux est située dans le tiers supérieur de cet hémisphère.

4. Un déplacement intracellulaire du plasme germinal survient entre les stades blastula et jeune gastrula.

5. Le nombre et la localisation des gonocytes primordiaux font penser qu'un de leurs noyaux n'aurait pu être transplanté que rarement au cours des recherches antérieures, et que de tels cas n'auraient probablement pas été décelables. Il est par contre très significatif que des noyaux provenant de cellules qui ne renferment pas de plasme germinal soient capables de donner naissance à des larves dont les gonades contiennent de nombreux auxocytes.

ACKNOWLEDGEMENTS

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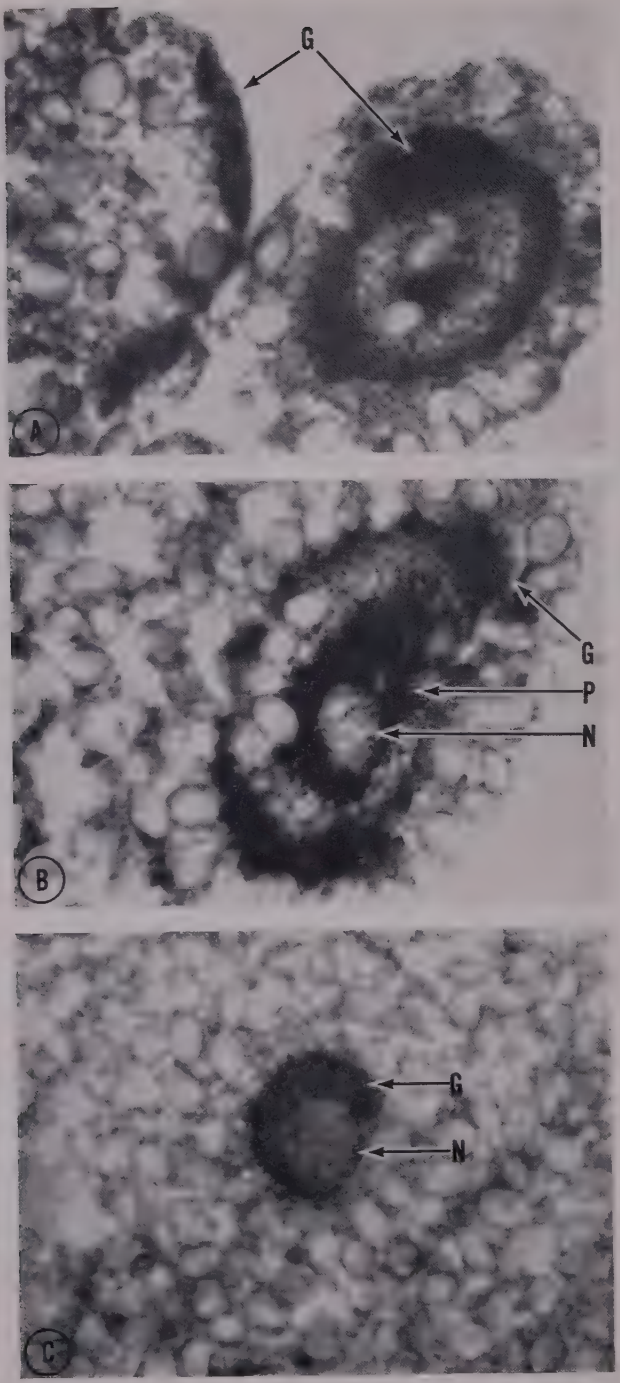
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EXPLANATION OF PLATE

Fig. A. Two cells from a mid-blastula stage of *R. pipiens*. Germ-plasm is present in the form of a ring in the cell on the right. The germ-plasm in the adjacent cell is located at the periphery of the cell in the form of an islet. ×666.

Fig. B. Cell from a mid-blastula stage of *R. pipiens*. ×666.

Fig. C. Cell from an early gastrula stage of *R. pipiens*. The germ-plasm is in close association with the nucleus and forms a cap around the nucleus. ×666.

G, germ-plasm; P, pigment; N, nucleus.

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The Two-gradient Hypothesis in Primary Induction. The Combined Effect of Two Types of Inductors Mixed in Different Ratios

by LAURI SAXÉN *and* SULO TOIVONEN[1]

From the Department of Physiological Zoology, University of Helsinki, Finland

WITH ONE PLATE

ON the basis of certain earlier suggestions made by Lehmann (1950) and Yamada (1950), together with our own experimental data, a modification of the two-gradient hypothesis of primary induction was presented by us some years ago (Toivonen & Saxén, 1955). Subsequently, this theory has often been referred to, accepted or criticized, and even misunderstood. There may thus be reasons for discussing it in the light of some recent experimental data.

At present there are limits to our opportunities of studying what is obviously the most important point in embryonic induction, the induction process itself. Simultaneously with such experiments on the induction process it is therefore necessary to continue research work on classical lines, and to obtain a further clarification of the causal relationships between the inductor and its morphogenetic action. A variety of qualitative investigations in this category have been made, but conceptions of the different quantities and the ratios of the active agents which participate in the primary induction are still based on indirect data. This is due to the lack of suitable methods for the testing of these compounds—which are still more or less hypothetical—in different quantities. A few attempts have been made in this direction, but the results are not quite conclusive (Shen, 1939; Niu, 1958): the complicated methods employed, the use of toxic materials, and the fractionation of inductively active samples may give rise to a variety of technical errors in experiments in which some chemical fractions are administered in different concentrations or amounts, either by adding them to the culture media or by incorporating them in inactive materials.

In the present experiments an attempt was made to approach the problem of the significance of the quantities of the inductors by employing the simplest possible method, i.e. by mixing two inductors with different inductive actions in different ratios, and then examining their combined action. The neatest way would have been to mix two chemical fractions of known inductive action, but the great number of unknown factors in such experiments (e.g. the degree of

[1] *Author's address*: Department of Physiological Zoology, Arkadiankatu 7, Helsinki, Finland.

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homogeneity of such mixtures, the release of the agents from certain materials used for their incorporation, &c.) made us decide to abandon this possible course of action. Instead, cells cultivated *in vitro* were used, both without pretreatment, and with a heat pretreatment known to inactivate the deuterencephalic and spinocaudal inductive action of the cells.

METHODS

HeLa-cells cultivated *in vitro* were chosen for the inductor material for two reasons. A homogeneous mixture of the cells could be expected according to preliminary results (Toivonen, Saxén, & Vainio, 1961) and according to certain control experiments (see below). Furthermore, the mixture could be used as such in implantation experiments without use being made of binding or incorporation material with an unknown effect on the process. Finally, the regulation of different quantities of the components in the experiments required no alterations in the size of the implant, which might otherwise have resulted in differences in the contact surface between the inductor and the reactive ectoderm. HeLa-cells had been used in a number of earlier experiments in our laboratory, and were known to be strong spinocaudal inductors (Saxén & Toivonen, 1957, 1958).

HeLa-cells were cultured in a standard culture medium containing 30 per cent. of human serum and 70 per cent. of Hanks's solution (see Saxén & Toivonen, 1958; E. Saxén & Penttinen, 1961). From our earlier experiments we knew that the spinocaudal inductive capacity of these cells was dependent on certain environmental factors, and especially on the pretreatment of the serum. In subsequent experiments we noted that during prolonged cultivation in the same, untreated, serum the inductive capacity of the cells changed (unpublished). Accordingly in the present experiments the culture medium was always changed 24 hours before using the cells, and only fresh, pooled human serum was used. In other respects the cultivation and the treatment of the cells did not differ from the methods employed in our earlier experiments (Saxén & Toivonen, 1958).

After 24 hours of cultivation in the fresh medium, cells from three Roux flasks were mechanically released, brought together, and centrifuged at 3,000 rev./min. The sediment was washed three times with Hanks's solution, and centrifuged after each rinse. Finally, the cells were suspended in 20 c.c. of saline, shaken to form a homogeneous suspension, and divided into two equal parts. The cells were counted from samples of these two halves. Subsequently, one-half of the cells was heated on a water-bath for 30 minutes at 70°, the other half being kept meanwhile in the culture incubator at 37°. These two cell suspensions were finally used for making the different mixtures of inductors.

Non-heated and heated cells were separately tested for their inductive effect. In addition, these two suspensions were mixed in the following ratios: 9 to 1, 7 to 3, 1 to 1, 3 to 7, and 1 to 9. The mixing of the cells in these experiments was performed as follows: both cell suspensions were shaken to a homogeneous suspension in 10 c.c. of saline. From these paired suspensions, 1 and 3 c.c. were

exchanged, the combined suspensions being repeatedly shaken and microscopically examined. In conclusion, each suspension was centrifuged for 10 minutes at 5,000 rev./min., the sediment being covered by cold, 70 per cent. alcohol and kept in a refrigerator for 4 to 8 hours at +4° C. After rinsing in Holtfreter's solution, small pieces were cut from the compact cell mass to serve as implantation material for 10 to 15 operations.

To check the degree of mixing of the cells, i.e. the homogeneity of the centrifuge sediment of a combined cell suspension, the following experiment was carried out. Before pretreatment, the culture media of HeLa-cells was transferred into Ringer's solution containing C-14 labelled algal protein hydrolysate (CFB-25, from the Radiochemical Centre, Amersham, England). After 6 hours of cultivation in this medium the cells were removed, suspended in 10 c.c. of saline, rinsed, and divivded into two halves. One-half was again heated as described above, the other half being kept meanwhile in the incubator. Simultaneously, the same quantity of cells was cultivated in non-labelled Parker's solution 199, and after division into two halves, treated like the labelled ones. Heated and non-heated cells from these two cultures were combined 1 to 1. Consequently two combined inductors were again obtained:

(*a*) 1 part heated, C-14 labelled cells to 1 part non-heated, non-radioactive cells;

(*b*) 1 part heated, non-radioactive cells to 1 part non-heated, C-14 labelled cells.

After centrifugation and alcohol treatment these suspensions were used as implantation material. The implanted embryos were fixed after 24 hours of cultivation at a late neurula stage, sectioned serially and covered by a stripping film (Kodak AR 10). After 3 days of exposure the films were developed and the distribution of labelled and non-labelled cells examined.

Young gastrulae of *Triturus vulgaris* were used as host animals in all the experiments here described. After implantation of the combined inductors the embryos were cultivated for 11 days in an incubator at 18° C. Serial sections were made from the larvae and they were stained and examined under the microscope.

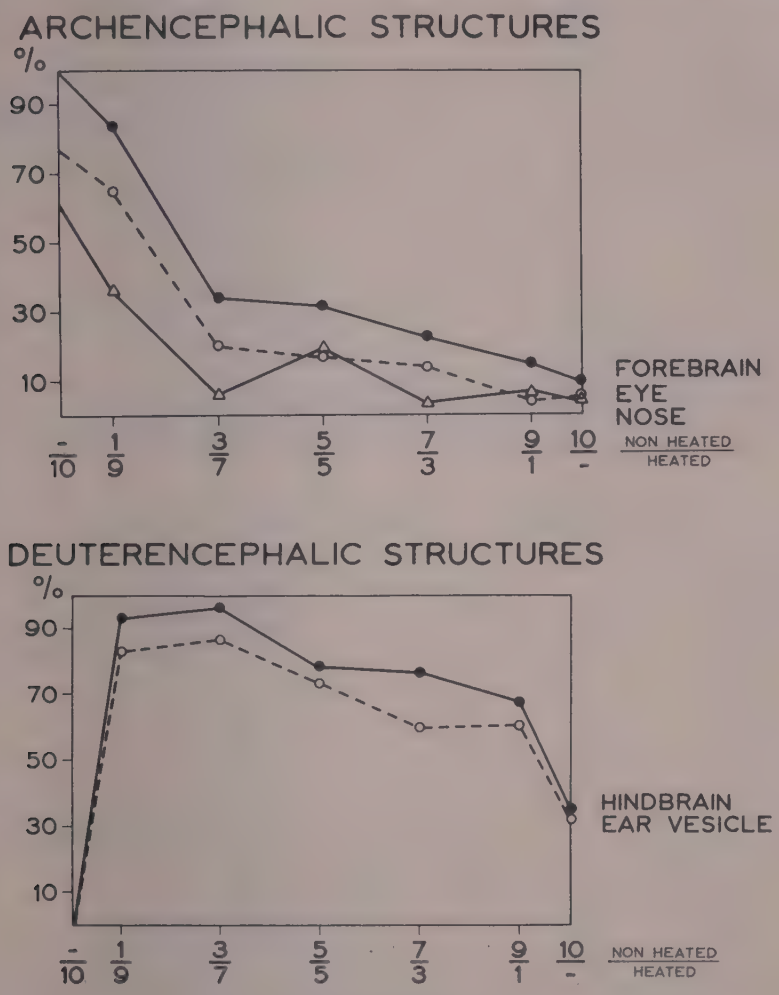
In the quantitative estimation of certain induced structures *camera lucida* drawings were used, made with a magnification of ×250. The drawings were made from complete series of 15 μ sections, 10 larvae in each series. To avoid the 'host influence' the larvae were selected according to the region of the secondary structures—all inductions here were located in the heart-liver region. Only the muscle tissue was drawn, and the total area of these drawings was measured by planimeter.

In each record of the secondary structures only the presence or absence of the structures was noted, and only in the case of muscle tissue was an attempt made to estimate the amount or volume of the tissues induced. In other words, the analysis of the series is qualitative in nature, and gives no information on the 'strength' of the induction.

RESULTS

Autoradiographic examination of the inductor

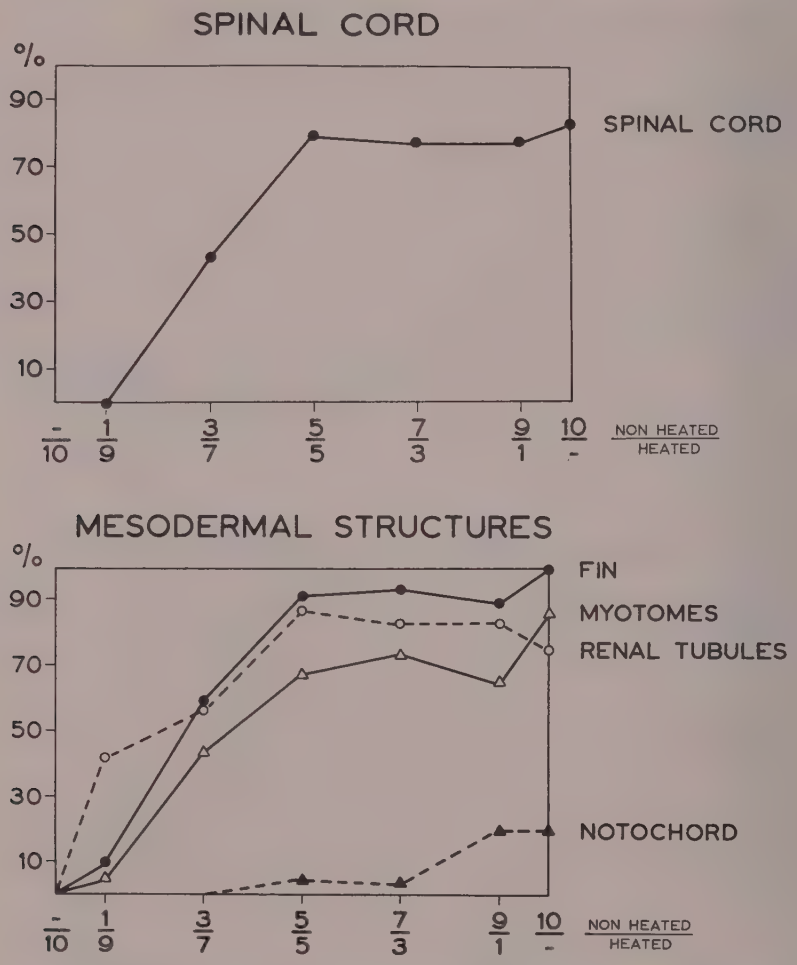
When a mixture of C-14 labelled and of non-radioactive HeLa-cells was used as inductor and the sections were covered by stripping film, 3 days'



TEXT-FIGS. 1 and 2. Incidence of archencephalic and deuterencephalic inductions in the experimental series.

exposure was sufficient for demonstration of the labelled cells in an autoradiograph. Examination of the autoradiographs (Plate) strongly hinted at an almost homogeneous mixture of heated and non-heated cells in the inductor.

After the 24 hours' cultivation of the host, only a very slight release of the labelled compounds was noted by employment of the present methods, and the non-labelled, killed cells obviously did not incorporate these labelled compounds. Hence, the two cell types were distinguishable, and showed a homogeneous mixture.



TEXT-FIGS. 3 and 4. Incidence of a secondary spinal cord and mesodermal structures in the experimental series.

Distribution of secondary structures in the series

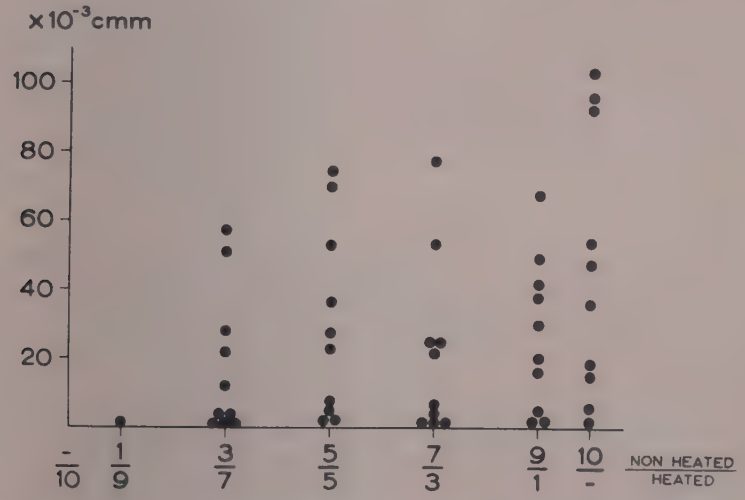
In each experiment about 50 operations were performed. Some embryos died during the subsequent cultivation, and some were malformed and discarded. The final results are based on the following number of larvae:

Non-heated cells	40	cases
Non-heated and heated cells, ratio 9:1	34	,,
,, ,, 7:3	35	,,
,, ,, 1:1	47	,,
,, ,, 3:7	39	,,
,, ,, 1:9	19	,,
Heated cells	44	,,

In all series the total induction percentage was 100. The distribution of archencephalic, deuterencephalic, and spinal structures, together with the incidence of mesodermal formations in the different series, is shown in Text-figs. 1–4. In addition, the original records of one series (1:1) are given in Table 1. These results are analysed below.

Quantitative estimation of the secondary muscle tissue

As already mentioned, the above results show only the qualitative analysis of the distribution of the secondary structures, as in most of the previous studies. Earlier, some authors have tried to estimate the volume of induced structures (Chuang, 1955; Nieuwkoop, 1958), and a similar approach was made in the present study. The different regions of the central nervous system can hardly be



TEXT-FIG. 5. Results of the quantitative determinations of the muscle tissue induced by different mixtures of cells.

used in such estimations because one cannot distinguish their borders in induced and often less organized formations. The mesenchyme also is not suitable for such calculations, and only occasionally was notochord induced in our series.

TABLE 1

The original records of the microscopical examination of 47 larvae induced by non-heated and heated cells mixed in ratio 1 to 1

Case No.	*Fore-brain*	*Eye*	*Nose*	*Hind-brain*	*Ear-vesicle*	*Spinal cord*	*Fin*	*Renal tubules*	*Myo-tomes*	*Noto-chord*
824				x	x	x	x		x	
825						x	x	x	x	
826						x	x	x	x	
827						x	x		x	
829				x	x	x	x	x	x	
832				x	x	x	x	x	x	
833						x	x	x	x	
834				x	x		x	x	x	
836						x	x	x	x	
837						x	x	x	x	
838				x	x	x	x	x	x	
841	x			x	x		x	x		
842	x	x		x	x	x	x	x	x	
843				x		x	x	x	x	
844	x	x	x	x	x			x		
845	x		x	x	x	x	x	x	x	
846				x	x	x	x	x	x	
847	x			x	x			x		
848						x	x	x	x	
849	x	x		x	x	x				
850	x	x	x	x	x		x	x		
851						x	x	x	x	
852	x			x	x	x	x	x	x	
853				x	x	x	x	x	x	x
855				x	x	x	x	x		
857				x	x	x	x	x		
858	x	x	x	x	x	x	x	x	x	
859	x	x	x	x	x	x	x	x		
860				x	x		x	x		
861	x			x	x	x	x	x		
862								x	x	
863	x		x	x	x	x	x	x	x	
864							x	x	x	
865				x	x		x			
866				x	x	x	x	x	x	
867	x		x	x	x	x	x	x	x	
868				x		x	x	x	x	
869				x	x	x	x	x	x	
870				x		x	x			
871							x			
873	x	x		x	x	x	x	x	x	
874				x	x	x	x	x	x	x
877				x	x	x	x	x	x	
879	x		x	x	x	x	x	x	x	
880				x	x	x	x	x	x	
881				x	x	x	x	x	x	
882				x	x	x	x	x	x	

Consequently, muscle tissue seemed to offer the only possibility for quantitative studies. The results are shown in Table 2 and Text-fig. 5. The variation in size of the secondary muscle formations was very great. In all series in which muscle

tissue was induced, there was observed a wide range from very small 'muscle fibres' to large masses of 50×10^{-3} mm.^{3} Simultaneously, with an increasing rate of muscle induction (Text-fig. 4), there seems to be a slight increase in the volume of this tissue. This can be seen as a decrease in the number of very small inductions, and as a slight increase in the mean volume.

TABLE 2

The total volume of the secondary muscle tissue in 10 larvae of the series 3/7, 5/5, 7/3, 9/1 and non-heated

In the series 1/9, only one muscle induction was noted, and none in the series with heated cells

	3/7	*5/5*	*7/3*	*9/1*	*non-heated*
	0·3	2·9	0·6	0·6	0·4
	0·8	3·2	0·6	1·2	6·5
	0·8	6·7	0·7	5·2	15·5
	0·9	7·9	2·4	16·0	17·8
	1·0	23·2	4·9	20·1	36·2
	12·3	28·0	21·9	29·6	47·3
	22·3	37·7	24·2	38·8	54·7
	28·6	53·1	24·3	42·6	93·9
	51·3	70·3	54·7	49·2	96·3
	58·2	75·3	76·8	68·8	104·1
Mean:	17·6	30·8	21·1	27·2	$47{\cdot}3 \times 10^{-3}$ cmm.

Thus the results of quantitative estimation of the 'muscle induction' may be summarized as follows: with an increasing rate of muscle induction the appearance of very small inductions seems to diminish, and simultaneously very large masses become rather more frequent. However, the differences are small, and in all the experiments complete series were noted, ranging from very small to relatively massive muscle inductions.

DISCUSSION

The first question for discussion is the homogeneity of the implanted material. Despite the fact that HeLa-cells, with and without pretreatment, were chosen as a suitable material in the 'mixing' of two inductors, we could not be sure that the two components of the final implantation material were homogeneously mixed to form one 'inductor' with evenly distributed active factors. If this was not the case, then the experiments would be comparable to our earlier implantation with two different inductors implanted simultaneously, but not mixed (Toivonen & Saxén, 1955). As stated above, every precaution was taken to maintain the homogeneity of the cell suspension during the various treatment stages, but there still existed the possibility that the heat pretreatment had changed the physical properties of the cells (e.g. their sedimentation rate, aggregation potentialities, &c.), and consequently that during centrifugation these cells would be separated from the non-heated ones.

As stated above, the control experiments with C-14 labelled cells suggested

that an almost complete mixing of the cells was obtained (Plate). The same conclusion can be drawn from the results. One complete experiment has been demonstrated in Table 1, and two points need stressing. First of all, no 'grouping' of certain types of induction can be noted despite the fact that on each occasion a new piece of the precipitated cell suspension was taken after about 10 implantations. If a fractionation by centrifuging had occurred, due to different physical properties of the cells, there would certainly have been layers yielding different inductive actions. Secondly, in all those cases showing archencephalic inductive action, i.e. the pure action of one of the mixed components, both deuterencephalic and spinocaudal formations were induced, which demonstrates the presence of the other component. From this it may be concluded that in different experimental series our implantation material represents a quite homogeneous mixture of the two inductors tested.

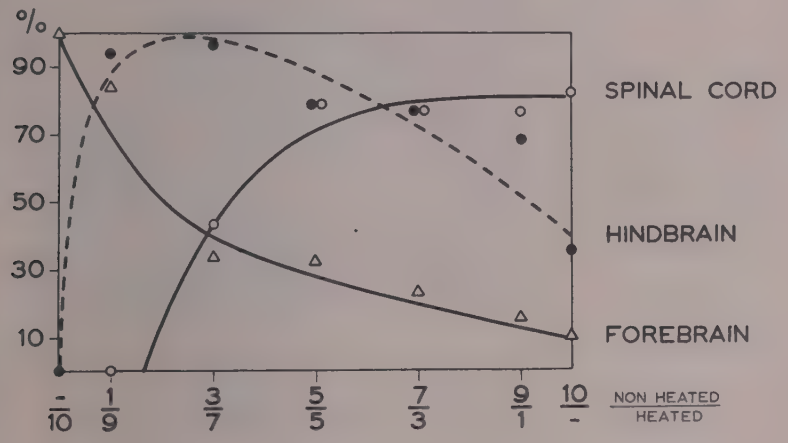
Nevertheless, the conclusion that we are obviously dealing with an almost homogeneous mixture of cells with different inductive actions does not mean that the implantation material can be considered as a chemical mixture of two or more inductively active agents. The differences are restricted to a 'cellular level', and in considering the suggestion that the inductive stimulus is mainly transmitted through the surface of contact between the inductor and the reactive ectoderm, it should be remembered that this surface of the implant is actually like a mosaic, the two cell types being obviously represented in a ratio which corresponds to the ratio on mixing. We return to this point below.

As mentioned in the introduction, the experiments were carried out in order to test our two-gradient hypothesis which suggests a neuralizing and a mesodermalizing principle. Before we embark further on these still hypothetical considerations, we have to repeat what can actually be read directly from the present results, and we shall only subsequently discuss them in the light of our theory.

The regional type of induction is usually divided into archencephalic, deuterencephalic, and spinocaudal, and some authors still suggest that these regions have their specific regional inductors (Tiedemann, 1959). In the present experiments we confirmed our earlier observations of the inductive action of HeLa-cells grown in fresh human serum (Saxén & Toivonen, 1958). They yielded a strong spinocaudal effect, and had a relatively weak deuterencephalic capacity. Furthermore, the well-known effect of short-term heat treatment was demonstrated. Following this treatment, the HeLa-cells induced purely archencephalic structures. In both series the induction rate was 100 per cent.

In mixing these two inductors with different regional inductive actions, a progressive shift of the regional character of the inductions was observed (Text-fig. 6)—obviously as a result of the combined action of the components. Starting from the purely archencephalic-inducing heated cells, even an addition of 10 per cent. of non-heated cells resulted in an induction of hindbrain structures to the extent of almost 100 per cent.; but it was hardly possible to detect the original action of the non-heated cells: renal tubules were frequently noted, but both

mesenchyme and myotomes were almost entirely absent, and spinal cord was never obtained. Thus the inductive action of this mixture is definitely a *new* one, and does not represent the regional inductive types of either of the two components of the mixture. This combined action is seen even more markedly in the next step, in which 30 per cent. of non-heated cells were added: all regional types are now represented in the inductions, but the dominating activity is a *deuterencephalic inductive action*. When heated and non-heated cells were mixed 1 : 1, this activity was more inclined towards a spinocaudal type, and when non-heated cells were added in increasing amounts the type of inductive action was progressively shifted towards the pure action of non-heated cells. This change is noted as a slight decrease in archencephalic and deuterencephalic inductions, accompanied by a slight increase in spinocaudal structures. In this connexion the appearance of notochord can be noted (Text-fig. 4).



TEXT-FIG. 6. The incidence of the three regional types of neural inductions in the experimental series.

Some of the above-mentioned results are, of course, quite as expected, and they are easily explained as a *competition of the reactive material* between the two inductively active components. For instance the progressive changes noted when the relative amount of spinocaudal inducing, non-heated cells was raised from 50 per cent. to 100 per cent., can thus simply be referred to as a 'masking' of the archencephalic action. However, this is not true as regards the deuterencephalic action. As emphasized above, the heated cells do not possess the capacity to induce hindbrain structures, and when non-heated cells were tested alone, deuterencephalic inductions were noted only in about one-third of the cases. Thus, the induction of a high percentage of deuterencephalic structures in series 1/9 and 3/7 must be due to a combined effect of archencephalic and spinocaudal actions. It may therefore be stated that in this instance the

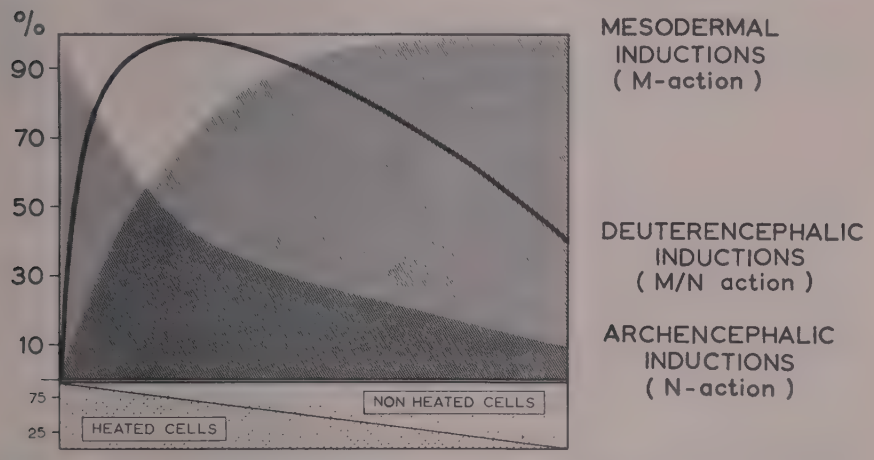
deuterencephalic inductor was experimentally built up—an observation which has to be discussed in the light of certain earlier data.

Yamada (1958, 1959) demonstrated a progressive shift in the inductive action of an originally purely mesodermalizing inductor when exposed to steam for various lengths of time. In this series there appeared a transitional deuterencephalic inductive action which disappeared after a prolonged heat treatment, to become a purely archencephalic action. The author was inclined to explain this as a progressive change in the protein molecule responsible for the inductive action. The present results, indicating that the same effect was obtained after an experimental combination of two killed inductor tissues, does not seem to support this suggestion. This might also be true of the earlier ideas of a specific inductor with deuterencephalic inductive action. Tiedemann (1959) isolated ribonucleoprotein samples yielding an almost pure deuterencephalic action, and seemed to believe in the possibility of the existence of a regionally specific deuterencephalic inductor. The present results may not definitely exclude such a possibility, but they do show that a similar effect can be obtained by mixing two inductors with different actions. Nevertheless, we are fully aware of one weak point in this argument; the deuterencephalic action is not completely lacking in the other component used in the mixtures, and consequently the definite rise of deuterencephalic structures might be explained as some kind of 'unmasking' of this action as a consequence of the increasing amounts of the heated cells, and not of a combined action. This possibility is dealt with below.

The two-gradient hypothesis

According to this hypothesis, not only is the deuterencephalic action discussed above a combined action of two active principles, but so also is the spinocaudal effect. We have termed these the neuralizing and the mesodermalizing principle; the former corresponds to the classical archencephalic inductors. Thus the effect of this principle is an induction of forebrain structures and the corresponding sense organs and placodes. The mesodermalizing action has never been demonstrated in a completely pure form, but certain heterogeneous inductors (such as guinea-pig bone-marrow, frog ventral skin) and protein fractions show a predominantly mesodermalizing action, accompanied only occasionally by the induction of small neural structures (Okada, 1948, Toivonen, 1953, 1954; Yamada, 1958, 1959; Tiedemann, 1959). In contrast, the neuralizing action can be obtained in pure form, e.g. after heat treatment of combined inductors (Toivonen & Kuusi, 1948). The combined action of these two factors has earlier been experimentally produced by us by the simultaneous implantation of tissues with neuralizing and almost purely mesodermalizing actions (guinea-pig liver+bone-marrow). As a result of this simultaneous implantation, an induction of spinal cord was noted in about 90 per cent. of cases as opposed to the series with pure bone-marrow in which the corresponding percentage was less than ten. At the same time the occurrence of hindbrain structures was

definitely increased compared with the action of liver and bone-marrow alone. If we now combine these series with the present observations with heated and non-heated HeLa-cells, there appears to be a continuous series from an almost pure mesodermal inductive action (bone-marrow) through spinocaudal (non-heated HeLa-cells and liver–bone-marrow combination) and deuterencephalic (series 3/7 and 1/9) to a pure neuralizing action (heated HeLa-cells). Very similar series were obtained by Engländer & Johnen (1957) after prolonged alcohol-treatment of a kidney tissue which originally induced spinocaudal structures, and by Yamada (1958, 1959) by short-term exposure to steam. Furthermore, our earlier series, in which HeLa-cells were cultivated in heat-inactivated serum for different periods of time, showed similar progressive



TEXT-FIG. 7. The incidence of archencephalic, deuterencephalic and mesodermal structures in the present experimental series.

changes in the inductive capacity (Saxén & Toivonen, 1958). We were inclined to explain this as a *progressive inactivation of the mesodermalizing principle* resulting in an 'unmasking' of the neuralizing principle—first combined with a weakened mesodermalizing action, and, finally, in a pure neuralizing action. If the present results are read 'from left to right', i.e. by starting from the neuralizing, heated cells, the result can consequently be explained as a *progressive increase of the mesodermalizing principle* (Text-fig. 7). Several earlier experiments with heat treatment suggest that the neuralizing principle is not affected by the 30-minute treatment at 70° (Toivonen & Kuusi, 1948; Kuusi, 1951). This idea apparently finds confirmation in the induction rate of 100 per cent. obtained in the present series with heated cells. Thus, when one takes into account that in all the present series the amount of HeLa-cells was roughly the same, it follows that the neuralizing principle was constant. Accordingly, the changes in the regional induction character must be due to changes in the labile mesodermalizing component. As a

consequence, it may be possible to state that the *regional inductive action of certain implanted tissues can be progressively altered by inactivation of the mesodermalizing principle in an originally combined inductor, but, vice versa, if a start is made from a purely neuralizing inductor the change can be obtained in the opposite direction by adding increasing amounts of this mesodermalizing principle to the inductor*. We are therefore inclined to feel that the present results, combined with earlier observations, give us strong evidence in favour of the two-gradient hypothesis.

As stated earlier, the hypothesis discussed here is a simplified model, and might even be viewed as an over-simplification (Dalcq, 1960). Actually, it is an attempt to explain the relation between the more or less hypothetical agents used in induction experiments and their inductive action. So far, it seems to explain such experimental data satisfactorily, but apart from the actual mode of their action, two more questions await an answer. The experiments were made under controlled conditions, using the same scheme of operations and the same material, and the role of the time factor and the competence of the reactive material may thus be excluded. Certain comments must be made, however. As shown by Johnen (1956) and Toivonen (1958), the induction process requires a definite time to occur, and according to the latter invesitgation it seems that the minimum time required is the same for both types of induction when killed tissues are used as inductors. However, Nieuwkoop has presented a hypothesis of the time factor in the induction process with which we are not in full agreement (Nieuwkoop *et al.* 1952; Nieuwkoop & Nigtevecht, 1954; Nieuwkoop, 1958). In this concept the differentiative stimulus starts with a general 'activation' which results in the formation of forebrain structures if no subsequent stimuli are present. This subsequent stimulation or 'transformation' leads to a regional segregation of the central nervous system and the induction of spinocaudal structures. If Nieuwkoop's 'activation' is termed 'neuralizing action', and his transformation 'mesodermalization', we are very close to our theory. The main difference, apart from the terminology, seems to be the time sequence of these two processes. According to Nieuwkoop, the preceding activation is a prerequisite for the subsequent transformation, and the present results might not exclude this possibility. However, one of the present authors has recently tested this hypothesis by employment of a somewhat different technique (Toivonen, 1961), and he has discovered that this sequence can be changed experimentally: the combined action of inductors was again tested. Heated, purely neuralizing HeLa-cells and mesodermalizing guinea-pig bone-marrow were used, not simultaneously as in the present series, but one after the other. The competent ectoderm was first induced by the bone-marrow for 3 hours, following which this inductor was *replaced* by the neuralizing heated HeLa-cells. In comparison with the effect of bone-marrow alone for 3 hours, and with the effect of the heated HeLa-cells, a combined action was again obtained. In other words, the ectoderm was first 'activated' into a mesodermal direction, and subsequently partly 'transformed'

into a neural direction. The observation does not seem to be in accordance with Nieuwkoop's idea of the temporal relationships of the induction process.

As regards the third component in the inductor–time–competence chain, the competence, Kuusi (1961) has recently presented an interesting idea. She points to the possibility that heat treatment known to 'inactivate' the mesodermalizing component can act in an indirect way by altering the physical properties of the inductor tissue. If the release of inductively active agents from the implant is delayed, the competence of the reacting tissue will change before these active compounds reach it. If the mesodermal competence is lost first, then the 'heat-inactivation' of the mesodermalizing principle could be explained in this way. However, certain earlier observations suggest that during normal gastrulation the neural competence is lost first (Holtfreter, 1938; Gallera, 1952; Nieuwkoop, 1958). As a result, much more experimental data are needed before this tempting hypothesis can be taken as proved. However, these two examples, the time factor theory of Nieuwkoop and the recent hypothesis of Kuusi, show us how complicated the study of the dynamic process of induction is.

Primary stimulus and subsequent interactions

At present our histochemical and submicroscopical methods do not allow us to follow the process of differentiation into neural versus mesodermal directions from the very beginning. Consequently, in all experiments which are concerned with the primary induction, the result of an inductive stimulus is not obtained until 7–12 days after the actual process has occurred. We know from the earlier experiments that a 3-hour contact with an inductor results in an inductive stimulus (Toivonen, 1958). From this it follows that the end-result after 11 days of cultivation, as, for instance, in the present series, must be a result of the action of the primary stimulus followed by a number of subsequent environmental actions. Thus our model of the effect of different ratios of mesodermalizing/neuralizing principle does not entail that the ectodermal cells, when stimulated by a definite 'mixture' of these agents, will be directly transformed into hind-brain or spinal cord cells, for instance, but that the relative *amounts of the primarily mesodermalized and neuralized areas respectively of the ectoderm are dependent on the M/N–ratio*. Their fate is determined on the basis of the relative amounts of these primarily determined cells. This seems to be dependent not only on the *relative amount* of these cells but also on their *absolute amount*.

These ideas are corroborated by a variety of experimental results. As regards the total amount of determined tissue, and its relation to the future fate of its cells, the results of Lopashov (1935) should be mentioned. He explanted fragments of presumptive head mesoderm of *Triturus* and obtained muscle differentiation only when the fragment was small, but notochord and brain vesicles were formed when several fragments were fused. Working with presumptive neural tissue of the mouse and chicken embryos Grobstein obtained a progressive decrease in the differentiation of the central nervous system when the

fragments were decreased from halves to one-sixteenths (Grobstein, 1952, 1955; Grobstein & Zwilling, 1953). From these results, Grobstein (1955) concluded that there 'appears to be a limiting minimum mass essential to continued morphogenesis and differentiation'. Muchmore (1957) used *Amblystoma* tissues in similar experiments, He explanted pieces of presumptive somite tissue from young neurulae, the smallest explanted piece corresponding to five presumptive somites. These small fragments differentiated only occasionally into muscle tissue, but when several such fragments were aggregated and explanted, muscle differentiation occurred in increased amounts. In addition, nephric tubules were differentiated more often, and certain other structures were also developed (neural formations, notochord, limb-buds). More recently Wilde (1961) demonstrated, in microdrop cultures of *Amblystoma* neuroepithelial cells, that a single isolated cell never differentiated, but that when two of these cells were cultivated in the same drop one of them would differentiate. Thus all these results demonstrate the "mass effect" under experimental conditions. It is tempting to suggest that similar masses are required for a manifestation of inductive stimuli in our experiments, i.e. that there is a *limiting minimum mass to be originally induced* for the manifestation of the differentiative stimulus. Thus a small amount of neuralizing principle cannot manifest itself when most of the cells are induced by the strong mesodermalizing principle present, as obviously in the experiments of Yamada (1959). Furthermore, the results of Muchmore (1957) might explain the old concepts of 'weak' and 'strong' mesodermalizing induction where the former leads to the formation of mesenchyme and nephric tubules, the latter to somites and notochord. This is also seen in our present results: nephric tubules are induced if 10 per cent. of 'mesodermalizing' cells are added, whereas myotomes need about 30 per cent. and notochord not less than 90 per cent. of these cells. In the light of the observations of Muchmore (1957), this could be explained as an induction of increasing amounts of ectodermal cells into a mesodermal direction. The same phenomenon might explain the somewhat unexpected results obtained in the quantitative estimations of the muscle tissue: if a comparison is made of Text-figs. 4 and 5, it may be noted that where an increase of the volume of secondary muscle tissue might be expected (series 7/3 to non-heated), this was hardly to be seen, but that simultaneously the incidence of notochord was increasing. Thus, a part of the originally mesodermalized ectoderm, instead of being differentiated into muscle tissue, was in fact differentiated into notochord, representing the 'strongest' mesodermalization.

We thus start from the concept that the first inductive stimulus will determine only the first step of differentiation of the ectodermal cells, i.e. the differentiation into either the neural or the mesodermal direction and probably also into the entodermal (Takata & Yamada, 1960). Due to the obvious mosaic-like distribution of the neuralizing and mesodermalizing 'units' on the contact surface of the implant, the overlying ectoderm will consequently be induced into intermixed islands of neural and mesodermal cells. The fate of these areas is then

determined by subsequent conditions. One of those essential factors which conducts the future development of these primarily induced cells might be the 'mass effect' mentioned above, leading to the lack of manifestation of the differentiative stimulus of very small areas. In addition, there are experimental data which demonstrate different 'inductive interactions' during the period of development following primary induction. For instance, the development of limb-buds might thus be correlated with the formation of nephric tubules (Muchmore, 1957), the differentiation of muscle tissue seems to be dependent on the presence of notochord (Yamada, 1940) and its growth enhanced by spinal cord (Holtzer, Lash, & Holtzer, 1956; Avery & Holtzer, 1958). In connexion with our present findings, the convincing experiments of Takaya are of special interest (Takaya, 1955, 1956*a*, 1956*b*). When explantation was made of the branchial part of the neural plate, the type of neural differentiation seemed to be dependent on the amount and the contact of mesenchymal and muscle tissue. In the total absence of these mesodermal components, the neural tissue developed into a vesicle resembling forebrain. When a larger amount of muscle tissue was in contact with the neural structures, these often resembled hindbrain structures, and ear vesicles were observed. In the presence of large amounts of muscle tissue, this often encircled the elongated neural tissue, which morphologically closely resembled the spinal cord. The author thus concluded that the regional character of the neural formations is not determined during the first step of induction, and that this is due to the neighbouring mesodermal structures. The results and ideas of Takaya thus seem to be fully in agreement with the two-gradient theory: a strong mesodermalizing action (M) combined with neuralizing action (N) was suggested as leading to differentiation of spinocaudal structures, and this seems to be the consequence of an induction of a large amount of mesenchymal tissue which determines the eventful fate of the simultaneously induced neural structures.

In the light of the ideas mentioned above, it might be interesting to compare the results of our earlier experiments (Toivonen & Saxén, 1955) with those of the present series. In the earlier series, the two different inductors were implanted simultaneously, but not intermixed, and thus the 'fields' of their inductive effects overlapped each other only partly, whereas in the present series no such individual fields could be expected. Consequently, in the liver–bone-marrow series we frequently obtained very complete inductions, closely resembling the dorsal side of the normal embryo. In these inductions, all the regional types of induction were represented, and usually in the normal sequence from pure archencephalic induction (liver effect) through deuterencephalic and spinocaudal structures (combined effect) to pure mesodermal masses (bone-marrow effect). In the present series, such complete inductions were seen only occasionally, and when archencephalic structures dominated, spinocaudal structures were usually absent.

To summarize the concepts embodied in this extensive and partly hypothetical discussion, it may be stated that the present experimental results, as well as a

variety of data obtained by other authors during the last five years, appear to corroborate our two gradient hypothesis (Toivonen & Saxén, 1955). Accordingly, these results can be explained by a primary neuralization versus mesodermalization by two different inductive principles, followed by a variety of interactions between these two tissue components, and leading to the regional type of induction noted under experimental conditions.

SUMMARY

The authors earlier presented a modified two-gradient hypothesis of primary induction, suggesting two inductively active principles. The regional type of induction would, according to this theory, be determined by the ratio of a neuralizing principle and a mesodermalizing principle. The present experiments were made in order to test this idea by the use of more quantitative methods.

HeLa-cells were used to produce, under experimental conditions, different ratios of the two principles in the implants. From earlier experiments it was known that these cells were, when cultivated in fresh human serum, strong inducers of spinocaudal structures, but after a short heat treatment, purely archencephalic inductors. Thus, cells with and without heat pretreatment were mixed in different ratios (1/9, 3/7, 1/1, 7/3, and 9/1). The inductive action of these cell mixtures was tested in the usual way in implantation experiments.

The results show that when non-heated cells are added to heated cells in increasing amounts, the regional type of induction produced by this mixture is progressively shifted from an archencephalic induction, through a deuterencephalic type, to a principally spinocaudal inductive action. Thus, the progressive shift from spinocaudal to archencephalic action earlier obtained by physical and chemical treatment of spinocaudal inductors, could be 'rebuilt' in the opposite direction.

The results are interpreted as corroborating the two-gradient theory. It is concluded that the primary inductive stimulus in these series led to determination of different amounts of neural and mesodermal cells, the final regional results being brought about by subsequent interactions of these two components. Certain recent findings which demonstrate a 'minimum mass effect' and late interactions between tissues are discussed.

RÉSUMÉ

L'hypothèse des deux gradients dans l'induction primaire. L'action combinée de deux types d'inducteurs mélangés selon des proportions différentes

Les auteurs ont présenté auparavant une hypothèse modifiée concernant l'existence de deux gradients dans l'induction primaire, suggérant la présence de deux principes actifs. Selon cette théorie, le type régional d'induction serait déterminé par le rapport entre un principe neuralisant et un principe mésoder-

misant. Les expériences rapportées ici ont été faites pour vérifier cette hypothèse au moyen de méthodes plus quantitatives.

On a utilisé des cellules HeLa pour produire dans les implants, dans des conditions expérimentales, de telles différences de rapport entre les deux principes. A partir d'expériences antérieures, on savait que ces cellules, quand on les cultive dans du sérum humain frais, sont de puissants inducteurs de structures spinocaudales mais, après un court traitement par la chaleur, sont des inducteurs purement archencéphaliques. Ainsi, des cellules après et sans prétraitement par la chaleur ont été mélangées selon diverses proportions (1/9, 3/7, 1/1, 7/3 et 9/1).

L'action inductrice de ces mélanges cellulaires a été testée de la manière habituelle par des expériences d'implantation.

Les résultats montrent que lorsqu'on ajoute en proportion croissante des cellules non-chauffées à des cellules chauffées, le type régional d'induction produit par le mélange passe progressivement d'une action inductrice archencéphalique à une action principalement spinocaudale, par l'intermédiaire d'un type deutérencéphalique. Ainsi, le passage progressif de l'action spinocaudale à l'action archencéphalique, obtenu antérieurement par traitement physique et chimique d'inducteurs spinocaudaux, a pu être reconstitué en direction opposée.

Ces résultats sont interprétés comme corroborant la théorie des deux gradients. On conclut que le stimulus inducteur primaire dans ces séries a conduit à la détermination de quantités différentes de cellules neurales et mésodermiques, les résultats régionaux étant finalement provoqués par des interactions ultérieures entre ces deux composants.

On discute certains résultats récents qui démontrent l'existence d'un 'effet de masse minimum' et d'interactions ultérieures entre les tissus.

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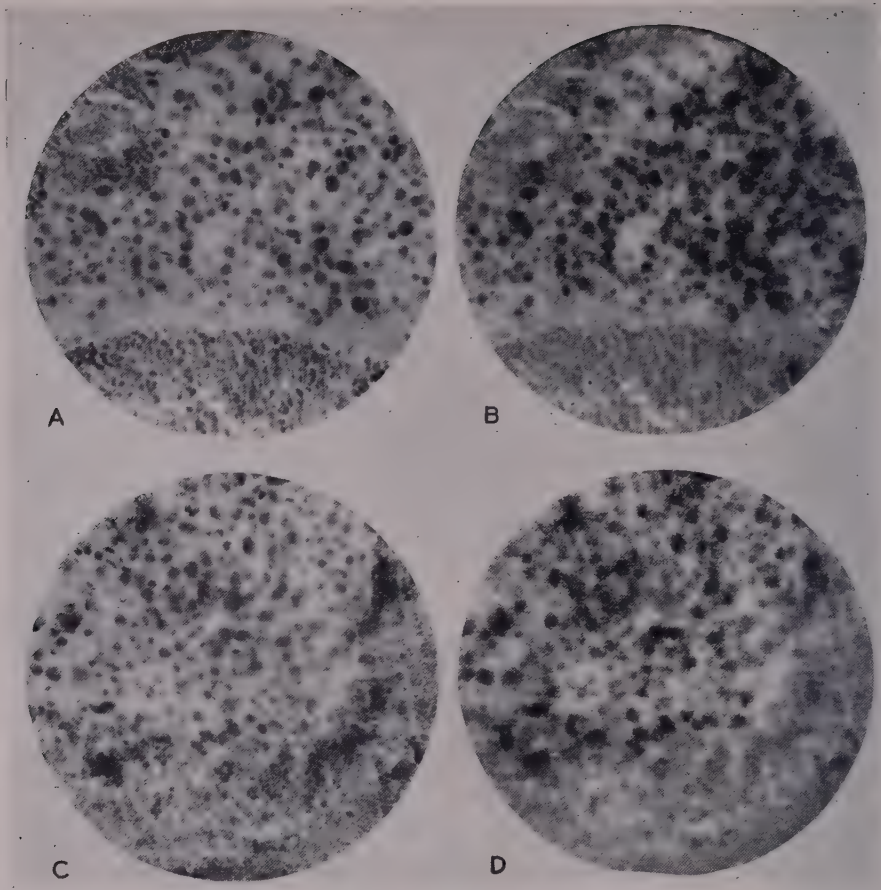

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EXPLANATION OF PLATE

Micrographs and autoradiographs of HeLa-cell mixtures after implantation and a subsequent cultivation of 24 hours. Stained with haematoxylin and eosin, autoradiographs by Kodak-AT-10 stripping film.

FIGS. A and B. 1 part heated, C-14 labelled cells to 1 part non-heated, non-radioactive cells.

FIGS. C and D. 1 part heated, non-radioactive cells to 1 part non-heated, C–14 labelled cells.

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The Differential Growth-response of Embryonic Chick Limb-bone Rudiments to Triiodothyronine *in vitro*

II. Growth Rate

by KIRSTIE LAWSON[1, 2]

From the Strangeways Research Laboratory, Cambridge

INTRODUCTION

THE maturation of the cartilage of embryonic chick long-bone rudiments growing in tissue culture is accelerated by addition of the thyroid hormones, thyroxine and triiodothyronine, but the growth in length of different long bones is not uniformly affected (Fell & Mellanby, 1955, 1956). Thus the growth of the hormone-treated tibia is less than that of a normal tibia, while the effect of thyroid hormone on the radius is to increase its growth. This differential response is not determined either by the stage of development at which the limb-bone rudiments are exposed to hormone, or by the size of the explant (Lawson, 1961).

Investigations to determine whether the differential response of limb-bone rudiments to triiodothyronine (T_3) is due to differences in the growth rates of different bones are described in this paper. The work was divided into three parts.

Data on the relative growth in weight of limb bones from the sixth to tenth day of embryonic development were obtained to supplement the data on the relative growth in length which were analysed by Landauer (1934) and Lerner (1936).

Growth *in vitro* can be altered by changing either the temperature at which the explants are incubated (Bucciante, 1926; Martinovitch, 1939), or the composition of the nutrient medium. The extent to which the growth and differentiation of chick skeletal explants are affected by the composition of the medium cannot be easily ascertained from previous studies on various media of biological origin (plasma and embryo extract: Strangeways & Fell, 1926; Miszurski, 1939; serum and embryo extract: Ito & Endo, 1956; plasma: Chen, 1954; serum: Hay, 1958) and on chemically defined media (Wolff, Haffen, Kieny, & Wolff, 1953; Biggers, Webb, Parker, & Healy, 1957; Kieny, 1958) since most of these studies have been made under different experimental conditions and with embryos of different ages. The results of a controlled comparison of the effects of different biological

[1] Sir Halley Stewart Junior Research Fellow.

[2] *Author's address*: Strangeways Research Laboratory, Wort's Causeway, Cambridge, U.K.

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media on the growth of limb-bone rudiments are described in the second part of the paper.

These studies of growth *in vivo* and *in vitro* provide a basis for the third part of the paper which consists of a description of the effect of T_3 on rudiments cultured under different growth-promoting conditions.

MATERIALS AND METHODS

Tissue culture

Media

Plasma and embryo extract. Limb-bone rudiments were grown on fowl plasma and chick embryo extract in watch-glasses (Fell & Mellanby, 1952) as previously described (Lawson, 1961).

Serum. For the study of growth in different biological media, serum was separated from cock plasma clotted with thrombin by centrifuging through a Hemming filter as described by Hay (1958). It was not necessary to incubate the plasma clot at 37° C. before or after breaking it up. For the other experiments, serum was obtained from a clot of cock blood which had been incubated at 37° C. overnight.

Broken clot. Plasma was clotted with thrombin or embryo extract in the upper bottle of a Hemming filter. The glass beads and rayon net were omitted from the upper side of the steel joint and the clot was broken as it passed through the perforated plate of the joint during centrifugation. This medium was transferred to the culture dishes with a 1-ml. syringe.

Chemically defined medium. CMRL 858 (Healy, Fisher, & Parker, 1955) or a similar medium, BL_1 (Biggers & Lucy, 1960), was used. The glucose concentration of both media was raised to 2·5 g./l.

Explants grown on serum, broken clot, or chemically defined medium were supported by lens paper (Chen, 1954).

3,5,3₄ triiodo-L-thyronine (T_3)

This substance was dissolved in 0·1 per cent. Na_2CO_3 or in 10 per cent. propylene glycol. The stock solution was added to plasma, serum, or the chemically defined medium to give a concentration of $1{\cdot}6 \times 10^{-4}$ g. T_3/l. of final medium, unless otherwise stated.

Volume of medium

The volume of medium and the proportions of explant to medium have already been described (Lawson, 1961). The medium used in the two factorial experiments in which the growth of bones on different biological media was measured, and in any experiment in which embryo extract was present, was obtained by mixing three volumes of plasma or serum with one volume of embryo extract or thrombin in 1 per cent. glucose Tyrode. In other experiments 80 volumes of serum were diluted with 1 volume of 16 per cent. glucose in distilled water.

Culture period

The explants were changed to fresh medium every other day and most experiments ended after 6 or 8 days.

Age of embryos

Rudiments were usually obtained from 6-day-old embryos (stage 28–29, Hamburger & Hamilton, 1951). In one experiment (see p. 545) rudiments were explanted 'at the same histogenetic stage': for convenience rudiments were selected at the stage when their ends were easily visible and their hypertrophic zones barely visible under the dissecting microscope, which in practice meant taking the femur and humerus from $5\frac{1}{2}$- or 6-day-old embryos, the tibia from 6-day-old embryos, the radius, ulna, and third metatarsus from $6\frac{1}{2}$-day-old embryos, and the third and fourth metacarpals from 7-day-old embryos.

Histological techniques

The rudiments were fixed in 3 per cent. acetic Zenker or in Rossman's fluid at 0° C. Paraffin sections were stained with Delafield's haematoxylin and chromatrope 2R or by the periodic acid/Schiff technique.

Measurement of the rudiments

Length, wet weight, and total nitrogen content were measured by methods previously described (Lawson, 1961). Total nitrogen was estimated on samples containing from 1 to 6 rudiments, according to size.

Experimental design

Paired samples

In experiments in which the effect of only one treatment was tested against a control, the rudiment from one side of the embryo was treated and compared with the untreated, corresponding rudiment from the other side of the same embryo.

Factorial experiments

The effects of several treatment combinations were compared in factorial experiments. The rudiments were allotted to the treatment combinations in a randomized block design. The data were analysed by analysis of variance procedures (Goulden, 1952) after transformation of the measurements to the common logarithmic scale. Significant reduction in experimental error was achieved by eliminating the effect of variations in the initial lengths of the bones from different embryos by an analysis of covariance (Biggers, Webb, Parker, & Healy, 1957).

Percentage growth in vitro

Total nitrogen was measured at the end of the culture period and compared with the initial total nitrogen of the corresponding rudiment from the same

embryo. The observations were transformed to logarithms so that the percentage increase (I per cent.) of any one rudiment was given by log I per cent. = log N_f—log N_i +2, where N_f = total nitrogen content of the rudiment at the end, and N_i = total nitrogen content at the beginning of the culture period. The mean log I per cent. for each type of bone was calculated and these values were used in statistical comparisons of the percentage growth of different bones. This procedure was adopted to equalize the variances of the percentage growth of the different bones. The theoretical aspects of the comparison of ratios have been discussed by Biggers (1961).

Estimation of response to T_3

The effect of T_3 was estimated from its effect on the final length and wet weight of the cultured rudiments, and on their growth in length (Lawson, 1961).

In experiments on paired samples, response was expressed as log T—log C, where T and C are the final length or wet weight of the treated and control rudiments. The significance of the difference in response of different bones was tested by the t-test for unpaired samples.

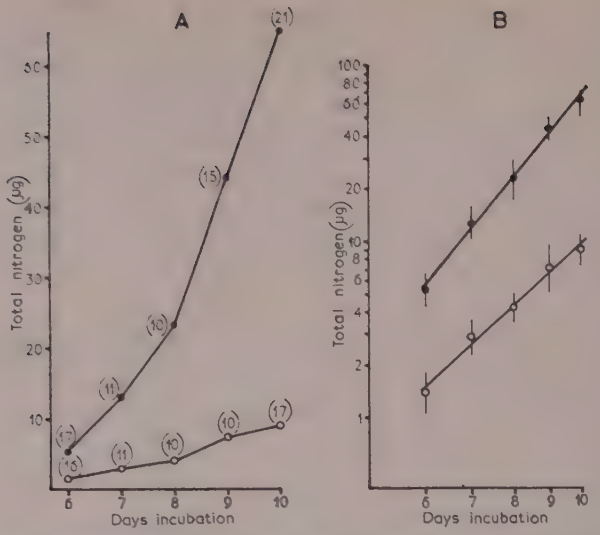
When the effect of T_3 was evaluated in a factorial experiment the data were transformed to the logarithmic scale, and the statistical significance of the treatments was tested by analysis of variance and covariance of the final length or wet weight of the rudiments and their initial length (see above).

RESULTS

The normal growth of limb-bone rudiments in vivo

The tibia and radius were chosen for the initial study of growth during the mainly cartilaginous phase of limb-bone development (6 to 10 days of incubation) because they show the largest difference in growth response to T_3 *in vitro*, and any growth differences that were related to their differential response might be more easily demonstrated. Furthermore, because these bones represent the two extremes in response, generalizations about their growth are likely to be valid for the growth of other limb bones, intermediate in response. The total nitrogen content of tibiae from 7- to 10-day-old embryos is highly correlated with the wet weight of the rudiments ($r = 0{\cdot}987$, $n = 25$); increase in total nitrogen was chosen as the criterion of growth of the whole rudiment because total nitrogen content can be determined more accurately than the weight of small samples. Seventy-four samples of tibiae and 64 samples of radii, obtained from 95 embryos, were estimated.

The results are plotted in Text-fig. 1A. The simplest expression relating size to time was sought which described the growth of different bones within the limits of experimental error and which could be expected to reveal growth differences between different bones. Linear regression analysis was done on the transformed values which, when plotted, appeared to lie approximately on a straight line and in which there was no significant heterogeneity of variance.



TEXT-FIG. 1. A, total nitrogen content of the tibia (dots) and the radius (open circles) from 6- to 10-day-old embryos. The number of samples estimated for each mean is indicated in brackets. B, the data from A plotted on a log-log scale and regression lines fitted. The vertical line through each mean represents twice the standard deviation. There is no significant heterogeneity in the variances.

TABLE 1

Results of analyses of variance and linear regression of total nitrogen of the tibia and radius from 6- to 10-day-old embryos

(*a*) *Analysis of log N with t*

Source of variation	Tibia		Radius	
	Degrees of freedom	*Mean square*	*Degrees of freedom*	*Mean square*
Linear regression	1	12·5912	1	6·1236
Deviations from linear regression	3	0·0631*	3	0·0534†
Error	69	0·0097	59	0·0124

(*b*) *Analysis of log N with log t*

Source of variation	Tibia		Radius	
	Degrees of freedom	*Mean square*	*Degrees of freedom*	*Mean square*
Linear regression	1	12·7290	1	6·2199
Deviations from linear regression	3	0·0171	3	0·0213
Error	69	0·0097	59	0·0124

* $P<0·001$. † $0·001<P<0·01$.

There was an approximately linear relationship when log nitrogen content (log N) was plotted against time (t), and also when log N was plotted against log t (Text-fig. 1B). Regression analysis, however, revealed significant deviation from linear regression for both bones when log N was analysed with t (Table 1*a*), but no significant deviation for either bone when log N was analysed with log t (Table 1*b*). In the latter analysis the value of the regression coefficient for the tibia, b_T, was 4·86, standard error 0·14, and this was significantly greater ($P<0{\cdot}001$) than that of the radius (b_R = 3·64, standard error 0·16). The difference between the regression coefficients is indicated by the difference in the slope of the two fitted regression lines in Text-fig. 1B.

The growth of both bones, for the 6–10-day period, can therefore be represented by the equation

$$\log N = \log a + b \log t, \tag{1}$$

$$\text{i.e. } N = at^b, \tag{2}$$

where a is a constant and b is the regression coefficient.

The specific growth rate is $(1/N)(dN/dt)$. By differentiation of (2)

$$\frac{1}{N}\frac{dN}{dt} = \frac{b}{t}.$$

Thus the specific growth rate decreases with time, but the ratio of the specific growth rates of the tibia and radius is constant, and equal to b_T/b_R. From the observations $b_T/b_R = 1{\cdot}33$.

If it is assumed that the growth of other limb bones can also be expressed within the limits of experimental error by equation (1), we have a convenient method for comparing the specific growth rates of different bones. Since the slope of a line may be estimated from two points on it,

$$b = \frac{\log N' - \log N}{\log t' - \log t},$$

where N' = mean total nitrogen content at time t' and N = mean total nitrogen content at time t.

The values of b for eight limb-bone rudiments—femur, tibia, third metatarsus, humerus, radius, ulna, third metacarpus, and fourth metacarpus, were therefore estimated from their total nitrogen content at 6 and 10 days. The total nitrogen content of the fourth metacarpus was measured also at 7 and 11 days, since at 6 days the rudiments consisted only of an ill-defined mass of procartilage. All the observations on 6-day-old embryos were made on samples containing several rudiments, because of the small size of individual rudiments; samples from 10-day-old embryos contained one or two rudiments. The regression analysis was weighted accordingly (Quenouille, 1958); the method is illustrated by the data for the humerus (Table 2).

The results of the nitrogen analysis and the calculated values of b are given in Table 3. The values of b for the tibia and radius in this series of eight bones are

slightly lower than those found when the whole of the 6- to 10-day period was sampled ($b_T = 4{\cdot}75$, cf. 4·86; $b_R = 3{\cdot}59$, cf. 3·64). The earlier analyses covered two years, and the measurements on the eight bones together were made during

TABLE 2

Data for weighted regression analysis of total nitrogen content of the humerus from 6- and 10-day-old embryos

6 *days* (*time t*)			10 *days* (*time t'*)		
X (*log N content/bone*)	w (*no. of bones/sample*)	wX	X	w	wX
0·616	6	3·696	1·585	1	1·585
0·911	4	3·644	1·364	2	2·728
0·790	6	4·740	1·581	2	3·162
0·688	6	4·128	1·508	1	1·508
0·701	6	4·206	1·672	1	1·672
0·693	6	4·158	1·449	1	1·449
0·690	6	4·140	1·651	1	1·651
0·698	6	4·188	1·508	1	1·508
0·693	6	4·158	1·460	1	1·460
0·590	6	3·540	1·374	1	1·374
0·582	6	3·492	1·518	1	1·518
0·666	6	3·996	1·614	1	1·614
	70	48·086		14	21·289

Weighted mean, $m_w = SwX/Sw = 0{\cdot}687$. $m'_w = 1{\cdot}521$.

$$b = \frac{m_w' - m_w}{\log t' - \log t} = 3{\cdot}76.$$

From weighted regression analysis, standard error of $b = 0{\cdot}21$.

TABLE 3

Total nitrogen content of limb bones from 6- to 10-day-old embryos

	'μg. total nitrogen						
	6 *days*	7 *days*	10 *days*	11 *days*	b	se_b	n
Tibia	4·85	—	55·41	—	4·75	0·26	24
Third metatarsus	1·14	—	10·67	—	4·58	0·28	24
Femur	6·08	—	48·76	—	4·08	0·22	24
Ulna	2·18	—	15·99	—	3·90	0·21	24
Humerus	4·86	—	33·16	—	3·76	0·21	24
Third metacarpus	1·06	—	6·71	—	3·60	0·26	24
Radius	1·32	—	8·28	—	3·59	0·25	24
Fourth metacarpus	0·62	0·72	2·35	3·45	3·42*	0·22	30

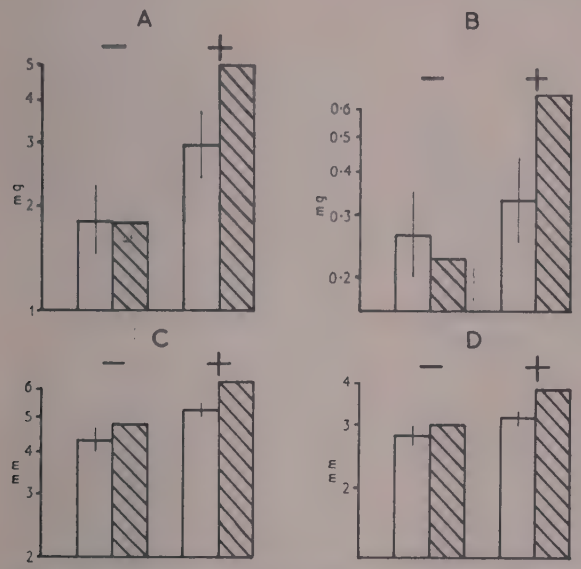
* The value of b for the fourth metacarpus was calculated from the total nitrogen content of bones from 7-, 10-, and 11-day-old embryos.

January and February; during these months the embryos are often smaller than later in the year and it may be inferred that growth is slower. Since the ratio of b_T/b_R in the original investigation was 1·33 and in the January/February results it was 1·32, it may be concluded that skeletal proportions were not affected.

Growth of limb-bone rudiments in vitro

The effect of different media on growth and differentiation

Tibiae and radii from 6-day-old embryos were grown on plasma or serum, with and without embryo extract. Four replicates each of eight bones were set up in a 2^2 factorial experiment, thus providing 32 samples of each type of bone for statistical analysis.

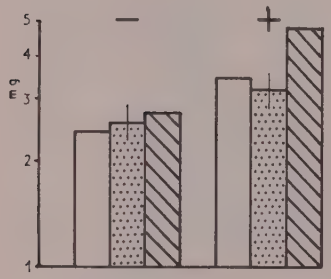


TEXT-FIG. 2. Adjusted means from the analysis of variance of the wet weight and length of the tibia (A and C) and of the radius (B and D) after 7 days in different biological media. In each figure the two columns on the left (−) show the size of rudiments cultivated without embryo extract, the columns on the right (+) show the size of rudiments cultivated in the presence of embryo extract. Open columns, serum; striped columns, plasma. Vertical lines represent 5 per cent. fiducial limits.

At the time of explantation each rudiment consisted of a cartilaginous rod with a crescent-shaped arrangement of cells towards the ends. The perichondrium had formed, but there was no osteoid tissue, and the central cells had not begun to hypertrophy, although they contained a little glycogen. At the end of the culture period all rudiments, except one on thrombin-clotted plasma, had developed the usual three zones of epiphysis, flattened cell zone, and hypertrophic shaft. Differentiation appeared to be more advanced in rudiments grown in the presence of embryo extract: the hypertrophic cells were larger and more osteoid tissue had formed than in rudiments grown in the absence of embryo extract. The periosteum of the rudiments grown on cock serum without embryo extract remained intact and healthy, unlike that of femora from 9-day-old embryos grown on horse serum without embryo extract (Endo, 1960). There

were no obvious histological differences between rudiments grown on plasma with embryo extract and those grown on serum with embryo extract, except that slightly more osteoid tissue was produced in the plasma cultures.

The different media affected the growth of the tibia and radius to the same extent. The growth of the rudiments was greatly enhanced by the presence of embryo extract in the medium and the effect of the embryo extract was greater when combined with plasma than with serum (Text-fig. 2). When embryo extract was replaced by an equivalent amount of Tyrode and thrombin there were no significant differences between the growth of rudiments supported by lens paper floating on serum and that of rudiments growing on the surface of the plasma clot (Text-fig. 2). It is therefore unlikely that the difference in the growth-promoting action of embryo extract in serum and in plasma is connected with mechanical differences in the two substrates. The most obvious explanation is that additional nutrients are provided by the proteolytic action of the embryo extract on the fibrin.



TEXT-FIG. 3. Adjusted means from the analysis of variance of the wet weight of the tibia after 6 days in different biological media. The three columns on the left (−) show the size of the rudiments cultured without embryo extract, those on the right (+) the size of the rudiments in the presence of embryo extract. Open columns, serum; dotted columns, broken plasma clot; striped columns, intact plasma clot. Vertical lines represent 5 per cent. fiducial limits.

This hypothesis was tested by comparing the growth of the tibia on three different media containing embryo extract: serum, plasma clot, and broken plasma clot (see section on Materials and Methods). Growth-promoting substances liberated from fibrin by the action of embryo extract would be expected to increase growth on the broken clot as compared with that on serum. Rudiments grown on the three media without extract served as controls. All components of the media were incubated at 38° C. for 1–2 hours before use, either as intact plasma clots in watch-glasses or as clots in the Hemming filter bottles before centrifugation. In the latter situation the plasma was either in contact with embryo extract (broken clot with embryo extract) or apart from it (serum with extract and the media without embryo extract). The six treatment combinations formed a 3×2 factorial experiment. A randomized block design of 18 units was used and the experiment was replicated once giving a total of 36 observations.

The final wet weight of the tibia was not enhanced by fibrin in a liquid medium of serum and embryo extract (broken clot) (Text-fig. 3, columns 4 and 5); there was increased growth ($P<0{\cdot}001$) only when the extract-containing clot was intact (Text-fig. 3, last column). The weight of the control rudiments grown on the media without embryo extract did not differ significantly. These results indicate

that interaction of embryo extract with the fibrin of the plasma does not enhance the growth of the cartilage rudiments; any additional growth-promoting substances liberated by digestion of the clot by embryo extract would be present in both the intact and the broken clot, but growth on the broken clot was no greater than on serum and embryo extract.

The fibroblastic outgrowth from the rudiments varied in different media and on different substrates and was removed when the medium was changed and before the rudiments were weighed. The outgrowth in the living cultures was

TABLE 4

Connective tissue outgrowth from tibiae cultivated on different media

		Without embryo extract			*With embryo extract*		
*Outgrowth**		*Serum*	*Broken clot*	*Clot*	*Serum*	*Broken clot*	*Clot*
2 days in culture	0	1	—	—	—	—	—
	+	5	6	—	6	3	—
	++	—	—	5	—	3	—
	+++	—	—	1	—	—	6
4 days in culture	0	4	5	—	2	—	—
	+	2	1	—	2	5	—
	++	—	—	5	2	1	—
	+++	—	—	1	—	—	6
6 days in culture	0	5	3	—	5	—	—
	+	1	3	—	1	3	—
	++	—	—	5	—	3	—
	+++	—	—	1	—	—	6

* 0, rudiment not attached to substrate. +, rudiment attached to substrate, but no fibroblastic outgrowth surrounding the rudiment. ++, rudiment firmly attached to substrate, outgrowth surrounding the rudiment. +++, abundant outgrowth.

scored on an arbitrary system (Table 4). By the end of the experiment there was little or no connective tissue outgrowth on lens paper when embryo extract was absent from the medium, although there was considerable outgrowth on plasma clotted with thrombin. Embryo extract maintained connective tissue outgrowth for a longer period when fibrin was present in the liquid medium (Table 4, columns 4 and 5); this suggests that fibroblastic outgrowth, but not cartilage growth (see above), is enhanced by the proteolysis of fibrin by embryo extract. There was abundant outgrowth on the intact plasma clot with embryo extract.

Differential growth in vitro

The growth rate throughout the culture period was not analysed, but the percentage increase in total nitrogen of various rudiments growing in different media was determined at the end of the culture period (Table 5) and the significance of the differences in percentage increase between bones was tested (Table 6).

The percentage increase in the total nitrogen content of small bones (ulna,

third metatarsus, radius, third metacarpus, and fourth metacarpus) varied after cultivation in plasma and embryo extract (Tables 5 *a*, *b*; 6 *a*, *b*). If these bones were arranged in order of their percentage growth, the sequence was the same as that of their specific growth rates *in vivo* (Table 3). Differential growth was also shown by the metatarsus and radius in a chemically defined medium during cultivation for 48 hours (Table 5*e*).

TABLE 5

Percentage increase in total nitrogen content of limb-bones in vitro

Conditions of experiment		*T*	*Mt*	*F*	*U*	*H*	Mc_3	*R*	Mc_4
(*a*) *p/e.e.*									
Bones from 28 6-day-old	Initial (μg.)	—	2·4	—	3·3	—	2·3	2·6	1·6
embryos *in vitro* for 6	Final (μg.)	—	16·0	—	17·6	—	9·4	7·3	4·1
days	% increase	—	594	—	442	—	321	198	156
(*b*) *p/e.e.*									
Bones at same initial	Initial	5·3	1·5	5·7	2·9	5·5	1·9	2·0	1·2
histogenetic stage, 8 of	Final	79·9	31·0	70·3	42·6	62·3	19·9	14·4	8·3
each *in vitro* for 8 days	% increase	1,397	1,926	1,127	1,375	1,042	905	720	585
(*c*) *p/e.e.*									
Bones from 4 7-day-old	Initial	13·5	—	—	—	—	—	3·0	—
embryos *in vitro* for 6	Final	67·5	—	—	—	—	—	24·4	—
days	% increase	400	—	—	—	—	—	741	—
(*d*) *serum*									
Bones from 9 7-day-old	Initial	13·5	—	—	—	—	—	2·9	—
embryos *in vitro* for 6	Final	37·4	—	—	—	—	—	5·3	—
days	% increase	177	—	—	—	—	—	83	—
(*e*) BL_1									
Bones from 48 7–8-day-	Initial	—	5·4	—	—	—	—	4·5	—
old embryos *in vitro* for	Final	—	7·5	—	—	—	—	5·8	—
2 days	% increase	—	41	—	—	—	—	30	—

ABBREVIATIONS: *p/e.e.*, plasma + embryo extract; serum, serum from whole blood; *T*, tibia; *Mt*, third metatarsus; *F*, femur; *U*, Ulna; *H*, humerus; Mc_3, third metacarpus; *R*, radius; Mc_4, fourth metacarpus.

The percentage growth of the tibia, femur, and humerus in plasma and embryo extract (Table 5*b*) was rather less than expected from the specific growth rates *in vivo*. Indeed, radii from 7-day-old embryos had a greater percentage increase than the corresponding tibiae when cultivated on this medium (Table 5*c*), though not when cultivated on serum (Table 5*d*). The growth of large explants may be limited by the ratio of surface area to volume, so that the more growth is promoted by the medium and the larger the initial size of the bone, the sooner a limiting value is reached. The variations in surface : volume ratio should not affect the relative growth of small bones, however, and differences in the percentage increase in size of such explants at the end of the culture period are considered to be a valid indication that bones differ in specific growth rate *in vitro* as well as *in vivo*.

TABLE 6

The significance of differences in percentage increase of total nitrogen of different bones in culture

Conditions of experiment		*T*	*Mt*	*F*	*U*	*H*	Mc_3	*R*	Mc_4
T	*b*	—	*	0	0	0	0	*	***
	c	—	—	—	—	—	—	**	—
	d	—	—	—	—	—	—	***	—
Mt	*a*	—	—	—	***	—	***	***	***
	b	*	—	*	**	**	**	**	***
	e	—	—	—	—	—	—	**	—
F	*b*	0	*	—	0	0	0	0	**
U	*a*	—	***	—	—	—	*	***	***
	b	0	**	0	—	0	0	*	***
H	*b*	0	**	0	0	—	0	*	**
Mc_3	*a*	—	***	—	*	—	—	**	**
	b	0	**	0	0	0	—	0	*
R	*a*	—	***	—	***	—	**	—	0
	b	*	**	0	*	*	0	—	0
	c	**	—	—	—	—	—	—	—
	d	***	—	—	—	—	—	—	—
	e	—	**	—	—	—	—	—	—
Mc_4	*a*	—	***	—	***	—	**	0	—
	b	***	***	**	***	**	*	0	—

Tests of significance were made on the data which are summarized in Table 5. Significant differences in the percentage growth between bones are indicated as follows: 0, $0{\cdot}05 < P$; *, $0{\cdot}01 < P < 0{\cdot}05$; **, $0{\cdot}001 < P < 0{\cdot}01$; ***, $P < 0{\cdot}001$. All possible comparisons were made within each group; significance at the 5 per cent. level should therefore be ignored. Experimental conditions (*a*-*e*) and abbreviations as in Table 5.

Growth and the differential response to T_3 in vitro

Relative growth and the differential response to T_3

The effect of T_3 was measured on different limb-bone rudiments explanted at the same histogenetic stage, because the response is modified by the stage of development at which the rudiment is first exposed to the hormone (Fell & Mellanby, 1956; Lawson, 1961). The rudiments were cultivated on clots of plasma and embryo extract. The percentage increase in total nitrogen was measured on similar rudiments from the same batch of embryos cultivated on control medium under the same conditions as the T_3-treated rudiments and their controls.

When the small bones, that is third metatarsus, ulna, radius, third metacarpus, and fourth metacarpus, were arranged in order of their response in T_3, the sequence was the same as when the bones were arranged in order of their percentage growth in normal medium, i.e. bones with the greatest percentage

increase in control medium were most retarded by T_3, while the growth of bones with a small percentage increase was stimulated by T_3 (Table 5*b*, cf. Table 7).

TABLE 7

The series of bones arranged in order of decreasing specific growth rate in vivo *compared with their response to T_3 after 8 days* in vitro

			Response n = 8*			
	b	*se*	*Wet weight*	*se*	*Length*	*se*
Tibia	4·75	0·26	−0·122	0·014	−0·092	0·009
Third metatarsus	4·58	0·28	−0·019	0·018	−0·061	0·014
Femur	4·08	0·22	−0·006	0·015	−0·076	0·005
Ulna	3·90	0·21	0·097	0·017	−0·015	0·007
Humerus	3·76	0·21	0·128	0·018	−0·048	0·009
Third metacarpus	3·60	0·26	0·236	0·033	0·043	0·006
Radius	3·59	0·25	0·327	0·030	0·059	0·012
Fourth metacarpus	3·42	0·22	0·331	0·049	0·070	0·021

* Response is expressed as the difference between the logarithms of the T_3-treated and the control bones.

The sequence of eight limb-bones when arranged in order of their response to T_3, was the same as their sequence when arranged in order of their specific growth rate *in vivo* (Table 7), i.e. T_3 retarded the growth *in vitro* of potentially fast-growing bones, such as the tibia and metatarsus, and increased the growth of bones, such as the radius and fourth metacarpus, which grow slowly *in vivo*.

Growth rate and the differential response to T_3

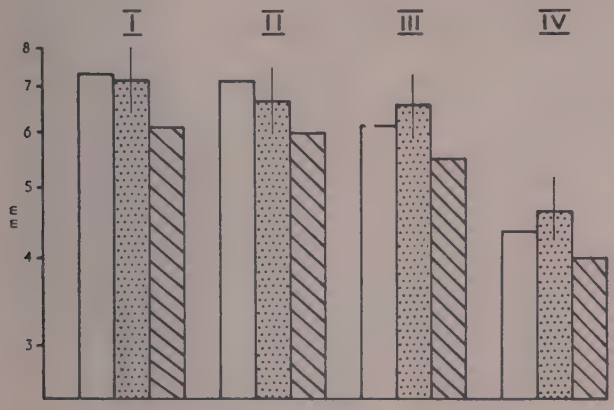
Since the differential response to T_3 appears to be associated with the specific growth rate *in vivo* and also with differential growth *in vitro*, the question arises whether the growth rate itself determines the response to T_3. If so, a bone that is retarded by T_3 in a medium in which it normally grows fast should be stimulated by the hormone when it is cultivated under conditions less likely to promote growth.

The effect of the medium on the response to T_3. The response of tibiae from 7-day-old embryos to two levels of T_3 (4×10^{-5} g./l. and $1{\cdot}6\times10^{-4}$ g./l.) was measured in four different media: plasma with embryo extract, serum with embryo extract, serum alone, and CMRL 858. The twelve treatment combinations made a 3×4 factorial experiment; this was replicated twice to give a total of 36 observations.

The final length of the bones treated with $1{\cdot}6\times10^{-4}$ g./l. was less than that of their controls in all the media tested (Text-fig. 4). The statistical interaction between the media and levels of T_3 did not approach the 5 per cent. level of significance; the apparent difference in response to the lower level of T_3 in different media was therefore a chance effect. Similar results were found when

the experiment was repeated with the femur and when the response was estimated on the wet weight of the rudiments.

Histological examination of the rudiments cultivated in the chemically defined



TEXT-FIG. 4. Length of the tibia after treatment with T_3 in different media for 8 days. I, plasma and embryo extract; II, serum and embryo extract; III, serum; IV, CMRL 858. Open columns, no added T_3; dotted columns, 4×10^{-5} g. T_3/l. medium; striped columns, $1{\cdot}6\times10^{-4}$ g. T_3/l. medium. Vertical lines represent 5 per cent. fiducial limits.

TABLE 8

The effect of T_3 on the final wet weight and total nitrogen content of the tibia and radius cultivated in serum for 6 days

(a) Tibia

	Control	T_3	*Response*†	*se*	*n*
Wet weight (mg.)	4·62	4·04	−0·052***	0·009	12
Total *N* (μg.)	44·5	38·5	−0·063*	0·017	6
$N/W\times100$	0·98	0·98	0·000	0·006	6

(b) Radius

	Control	T_3	*Response*†	*se*	*n*
Wet weight (mg.)	0·36	0·63	0·224***	0·033	11
Total *N* (μg.).	6·2	9·0	0·167*	0·049	5
$N/W\times100$	1·49	1·23	−0·084**	0·001	5

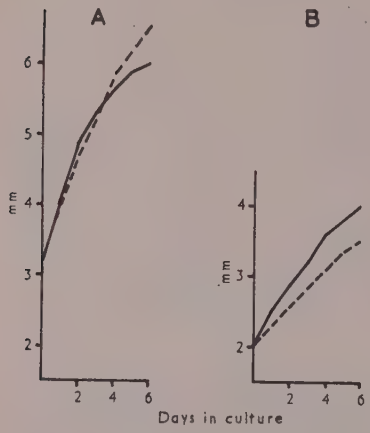
* $0{\cdot}01<P<0{\cdot}05$. ** $0{\cdot}001<P<0{\cdot}01$. *** $P<0{\cdot}001$.

† Response is expressed as the difference between the logarithms of the T_3-treated and the control bones.

medium showed a localized necrosis in the flattened cell zones of the T_3-treated rudiments. The formation of matrix was deficient throughout the rudiments in both control and treated explants.

The response to T_3 in serum was examined in more detail. Twenty-four pairs

of tibiae and radii from 7-day-old embryos were cultivated on serum from whole blood for 6 days. The concentration of T_3 added to the treated cultures was $1{\cdot}6\times10^{-4}$ g./l. of medium. The growth in length of the tibia was slightly stimulated by T_3 during the first 24 hours ($P<0{\cdot}01$) (Text-fig. 5*a*). The characteristic retardation appeared in the T_3-treated rudiments after the third day in culture. The final wet weights and total nitrogen contents of the T_3-treated tibiae were also less than those of the controls (Table 8). Thus, although the percentage growth of the control tibia in serum was less than that of the control radius in plasma and embryo extract (Table 5 *c*, *d*) the growth of the tibia was retarded by T_3 in both media. The growth in length of the radius was stimulated by T_3 during the first 4 days in culture. During the last 2 days the length of the T_3-treated rudiments increased at the same rate as that of the controls (Text-fig. 5*b*). Both the final wet weight and total nitrogen content were greater in the T_3-treated radii than in the controls (Table 8).



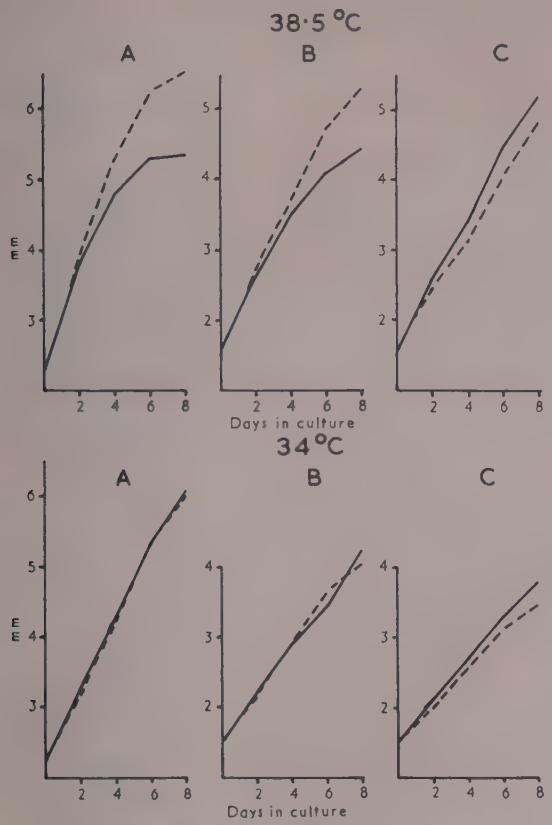
TEXT-FIG. 5. Growth in length of the tibia (A) and the radius (B) during cultivation for 6 days on serum. Broken lines, the length of the control bones; continuous lines, the length of the equivalent rudiments grown in the presence of $1{\cdot}6\times10^{-4}$ g. T_3/l.

The effect of temperature on the response to T_3. Tibiae, third metatarsi, and radii from 6-day-old embryos were grown in plasma and embryo extract for 8 days. The response to T_3 at different temperatures was tested on 24 bones of each type in a 2^2 factorial experiment in which the factors were T_3 (control and $1{\cdot}6\times10^{-4}$ g. T_3/l.) and incubation temperature (34° C. and 38·5° C.).

The rudiments appeared healthy and of normal shape after growth at both temperatures.

At 34° C. the retardation of growth in length, characteristic of the leg bones cultivated in T_3 medium at 38·5° C., did not occur, but there was no stimulation of growth (Text-fig. 6 A, B). The growth in length of the radius, however, was stimulated both at 34° C. and at 38·5° C. (Text-fig. 6C). This stimulation was significant at the 1 per cent. level.

The growth in length of the control bones was considerably reduced at 34° C. when compared with growth at 38·5° C. ($P<0{\cdot}001$), but relative differences in growth were maintained. For example, the metatarsus grew 3·8 mm. at 38·5° C., but only 2·5 mm. at 34° C.; the radius, which was the same initial length as the metatarsus, grew 3·3 mm. at 38·5° C. compared with 1·9 mm. at 34°. Thus even though the metatarsus grew more slowly at 34° C. than the radius at 38·5°, its growth was not stimulated by T_3.



TEXT-FIG. 6. Growth in length of the tibia (A), third metatarsus (B), and radius (C) during 8 days cultivation at 38·5° C. (upper figures) and at 34° C. (lower figures). Broken lines, the length of control bones; continuous lines, the length of bones treated with T_3.

DISCUSSION

The normal growth of limb-bone rudiments in vivo *and in culture*

No biological significance can be attached to the exact analytical form of the growth equation (Gray, 1929; Kavanagh & Richards, 1934; Medawar, 1940, 1945); it is useful in so far as it makes the experimental results available in an algebraic form from which information concerning the specific growth rate, which is considered to have biological significance, can be extracted easily. The power law $N = at^b$ was chosen because it fitted the data of both the tibia and the radius within the limits of experimental error; these limits were sufficiently narrow for statistical control to reject the exponential formula, $N = ab^t$. Since a significant proportion of the total nitrogen of the rudiments must be contributed by the intercellular matrix which is not self-reproducing, this result

is satisfactory. The growth equation implies that the specific growth rates of chick limb-bone rudiments during a restricted period of their normal development, decrease with time; this finding is neither original nor unexpected, but the formula indicated a convenient method for comparing the specific growth rates of different bones.

The faster rate of growth in length of the long bones of the leg as compared with the growth rate of the wing bones (Landauer, 1934) is reflected in the whole limb by the faster rate of growth in weight (Schmalhausen, 1926) and in DNA content in the hind limb (Nowinski & Yushok, 1953). Lerner (1936) derived values from Landauer's (1934) data for the growth ratio of bone length to body-weight in the allometry equation $y = bx^{\alpha}$, where y and x are magnitudes relating to part and whole respectively, and α is the growth ratio (Huxley & Teissier, 1936). The descending order of growth ratios was metatarsus, tibiotarsus, femur, humerus, radius, ulna. The distal-proximal growth gradient in the leg was also found in Creeper embryos and in other experiments (Lerner & Gunns, 1938). Since the allometry formula implies that the ratio of the specific growth rates of the parts compared is constant, the sequence of bones arranged in order of their specific growth rates, inferred from the values of b calculated from total nitrogen content, should be the same as the sequence of their specific growth ratios. Although the total nitrogen content of the three leg bones which were investigated increased faster than that of any of the wing bones, there was no obvious growth gradient in the leg. It is possible that the measurement by Lerner of the combined tibia and tarsus and the relative thickness of different bones are factors in the difference in sequence. For example, Lerner found that the value of α for the radius was slightly greater than that for the ulna, but in the present work the reverse was found. Similarly, the value of b for the third metacarpus was considerably higher than that of the fourth metacarpus.

Relative growth *in vitro* was inferred from differences in the percentage increase of total nitrogen of explanted small rudiments; the pattern of relative growth in various media was the same as *in vivo*. The difference in the relative growth in length of the metatarsus and radius was also maintained when the growth rate was reduced by lowering the incubation temperature. These results agree with the findings of Lerner & Gunns (1938) who compared the relative growth rates in the leg bones in 11- to 18-day-old embryos incubated at 98°, 101°, and 104° F. There was increased growth at the higher temperatures, but the values of α were unaffected and so normal proportions were maintained. It is concluded that the relative growth of different bones during normal development is intrinsic by 6 days of incubation, when the bones have differentiated as distinct cartilaginous rudiments, and is not imposed by environmental factors in the limb. The metabolic basis of the proportionate growth of the skeleton remains unexplored; the establishment of regional differences within the limb occurs at an early stage in the development of the limb-bud, and may be controlled by the apical cap (Saunders, Cairns, & Gasseling, 1957).

The experiments on the growth of rudiments in different biological media showed that growth was greater when embryo extract was added to plasma than when it was added to serum, but that growth was similar on plasma and serum in the absence of embryo extract (Text-figs. 2, 3). Peptic digests of fibrin slightly enhance the growth of fibroblasts on plasma clots (Carrel & Baker, 1926, Willmer & Kendal, 1932), and in this laboratory the exudate from an incubated plasma-embryo extract clot is used as a better growth-promoting medium for fibroblasts in hanging drop cultures than serum and embryo extract. The results of the experiment with broken plasma clots show that any substance which may be produced by the proteolysis of fibrin by embryo extract, if present in addition to serum and embryo extract, do not increase the growth of the whole rudiment. The enhanced organized growth of the bone rudiments on the plasma and embryo extract medium as compared with that on serum and embryo extract, appears to be associated with the abundant fibroblastic outgrowth in these cultures; the reason for this is not clear.

The differential response to T_3

The differential growth response of different rudiments to T_3 is not dependent on the absolute growth rate of the rudiments *in vitro*, whether this is controlled by the composition of the medium or by the incubation temperature. Neither of these methods of controlling growth rate disrupt the pattern of relative growth and the differential response to T_3 *in vitro* appears to be closely associated with the relative growth of the rudiments *in vivo* (Table 7).

Of the bones studied, the tibia and radius show the greatest difference in their growth response to the same concentration of T_3 *in vitro*. The drastic reduction in the growth of the tibia was modified in three situations. The growth in length of T_3-treated tibiae from 5- to 5½-day-old embryos was slightly stimulated during the first 2 days in culture (Lawson, 1961); this was interpreted as a precocious onset of hypertrophy. A transitory stimulation of growth in length also occurred in tibiae that were cultivated on serum (Text-fig. 5); these rudiments were taken from 7-day-old embryos and hypertrophy had already begun in the shafts. T_3 did not modify the growth in length of tibiae cultivated on plasma and embryo extract at 34° C. (Text-fig. 6). The increase in the growth in length of the T_3-treated radius, both in serum and at 34° C., was proportionately greater than that produced at 38° C. by the same concentration of hormone added to plasma and embryo extract. The present results are not necessarily inconsistent in view of the differences in sensitivity of different bones to T_3 which will be demonstrated in a future paper. The effective concentration of T_3 added to serum may have been lower than in plasma and embryo extract as the natural thyroid hormone of the serum may have been destroyed during the incubation of the blood-clot overnight at 37° C. (see section on Materials and Methods); in the experiments at subnormal temperature, T_3 added to plasma and embryo extract may have been less active at 34° C. than at 38° C.

Fell & Mellanby (1955, 1956) found that the length of the flattened cell zone was reduced by treatment with thyroid hormone; the amount of reduction varied, but was greatest in the leg bones. They interpreted this as precocious hypertrophy in the flattened cell zone without an accompanying increase in cell-division. Experiments on chemically defined media (Kieny, 1958) showed that the process of hypertrophy and lengthening of the shaft occur without any increase in dry weight of the rudiment; it is possible that the increased growth in length of the radius in response to T_3 may have been due to such a process. On the other hand, the few measurements that were made of the effect of T_3 on the total nitrogen content of the rudiments (Table 8) indicate that T_3 increases the synthesis of nitrogenous compounds in the radius. The ratio of nitrogen to wet weight of the radius is reduced by treatment with T_3, however, so an increase in water content cannot be excluded.

The warning of Weiss (1949) against considering growth and differentiation as separate phenomena in embryogenesis is relevant; during the course of skeletal differentiation the speed of a synthetic process varies. For example, the young hypertrophic cells are the most active incorporators of S^{35} (Amprino, 1954; Fell, Mellanby, & Pelc, 1956); hastening the maturation of these cells, therefore, would limit chondroitin sulphate synthesis and might result in an apparent retardation of the growth of the whole cartilaginous rudiment. The results of the present experiments on the growth of the rudiment as a whole, serve to emphasize the differences in behaviour between various rudiments and to indicate that the response to T_3 probably depends on differences in metabolism that are reflected in the relative growth of different rudiments; the study provided no indication of the nature of the metabolic basis of these differences.

SUMMARY

1. The growth of the tibia and radius from 6- to 10-day-old embryos was estimated from the increase in the total nitrogen content of the rudiments. A comparison of the specific growth rates of eight limb bones showed that the bones could be arranged in the following order of diminishing specific growth rate: tibia, third metatarsus, femur, ulna, humerus, third metacarpus, radius, fourth metacarpus.

2. If the small bones were arranged in order of the percentage growth of their rudiments *in vitro*, the sequence found in various media was the same as that of their specific growth rates *in vivo*.

3. A comparison of the growth-promoting properties of various biological media showed that the best growth and differentiation occurred on a plasma and embryo extract clot. The growth-promoting properties of the extract were greatly enhanced in a plasma clot with which the fibroblasts of the explant, through their abundant outgrowth, were in intimate contact. In the absence of extract there was no difference between the growth of rudiments in contact with the clot or supported by lens paper floating on serum.

4. The order of a series of bones arranged according to their growth response to T_3 in a medium of plasma and embryo extract was the same as their order when arranged according to their specific growth rate *in vivo*: the growth of potentially fast-growing bones was reduced by T_3, that of slowly growing bones was increased.

5. The growth rates *in vitro* of different rudiments were varied by altering either the composition of the medium or the incubation temperature, but the rudiments continued to respond differentially to T_3. The characteristic differences in the relative growth of the different bones when not treated with T_3 were maintained under these conditions.

RÉSUMÉ

Action de la triiodothyronine sur la croissance des ébauches cartilagineuses de différents os longs

II. Vitesse de croissance

1. Pour estimer la croissance du tibia et du radius d'embryons de poulets de 6 à 10 jours d'incubation, on a étudié la teneur en azote total de ces ébauches. En comparant la vitesse de croissance ($1/N.dN/dt$) d'ébauches cartilagineuses de 8 os longs on peut les classer, selon un ordre décroissant: tibia, 3ème métatarsien, fémur, cubitus, humérus, 3ème métacarpien, radius, 4ème métacarpien.

2. On retrouve la même séquence lorsqu'on cultive *in vitro* les ébauches de ces différents os sur des milieux variés.

3. Les meilleures croissance et différenciation se réalisent sur des coagula à base de plasma et d'extrait embryonnaire. Les propriétés stimulatrices de l'extrait embryonnaire sont nettement renforcées lorsque les fibroblastes de l'explant, à la suite d'une abondante prolifération, sont en contact intime avec le coagulum. En l'absence d'extrait embryonnaire il n'y a aucune différence entre la croissance des ébauches cartilagineuses cultivées en contact direct avec le coagulum ou déposées sur du 'lens paper' flottant dans du sérum.

4. L'addition de triiodothyronine (T_3) dans le milieu qui contient du plasma et de l'extrait embryonnaire, d'une part, inhibe la croissance des os à vitesse de croissance élevée *in vivo*, d'autre part, stimule la croissance des os à vitesse de croissance basse *in vivo*.

5. Les différences caractéristiques de la vitesse de croissance des différents os longs sont maintenues lorsqu'on modifie la composition du milieu ou la température d'incubation. Mais, même dans ces conditions les ébauches cartilagineuses continuent de répondre différemment à la présence de T_3 dans le milieu.

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Malformations résultant d'irradiations localisées de différentes parties de l'ébauche cardiaque chez l'embryon de Poulet

par GEORGES LE DOUARIN[1]

Laboratoire d'Embryologie expérimentale du Collège de France et du C.N.R.S., Paris. (Directeur: Professeur Ét. Wolff)

AVEC UNE PLANCHE

L'IRRADIATION aux rayons X de la totalité de l'ébauche cardiaque, pratiquée sur l'embryon de Poulet au 3ème jour de l'incubation, provoque des malformations cardiovasculaires qui, en ce qui concerne le cœur, intéressent la morphologie externe de l'organe ainsi que sa constitution interne. Ces résultats ont été décrits dans un travail antérieur (G. Le Douarin, 1960).

Pour expliquer la genèse de ces malformations, on peut envisager d'une part la radiodestruction d'une certaine quantité de matériel de l'ébauche cardiaque — donc un effet direct du rayonnement — et d'autre part des perturbations de la physiologie cardiaque qui sont la conséquence de la radiolésion. Un effet secondaire de l'irradiation du cœur se manifeste ainsi au niveau des arcs aortiques dont l'évolution normale est fréquemment perturbée, fournissant alors des systèmes artériels aberrants (G. Le Douarin & N. Le Douarin, 1959). On connaît par ailleurs l'importance des courants sanguins dans le modelage des cavités cardiaques et dans la réalisation d'un cloisonnement normal (Bremer, 1928).

L'irradiation localisée de certaines parties de l'ébauche cardiaque au 3ème jour de l'incubation a été réalisée pour tenter de préciser le mécanisme de l'apparition de certaines défectuosités du cœur obtenues après l'irradiation totale de cette ébauche. La méthode utilisée dans ce travail est celle qui a été mise au point par Ancel & Wolff (1934).

Le rayonnement est fourni par un appareil Picker-X-Rays, à anticathode de tungstène de 4 mm.², fonctionnant sous une tension de 60 kV. et à une intensité de 8 mA. ou de 15 mA. selon les séries expérimentales. La distance de l'anticathode à l'embryon est de 13 cm. Le tube localiseur employé absorbant environ 30 pour cent du rayonnement, le débit utile est de l'ordre de 500 r/minute lorsque l'intensité est de 8mA. et de 820 r/minute lorsqu'elle est de 15 mA.

[1] *Author's address*: Laboratoire d'Embryologie expérimentale du Collège de France, 49bis, Avenue de la belle Gabrielle, Nogent-sur-Marne, Seine, France.

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RÉSULTATS

Irradiation du bulbe et du tronc artériel (Planche, fig. A)

Séries expérimentales

Les séries expérimentales réalisées sont représentées sur le tableau 1.

TABLEAU 1

Dose	*Stade de Hamburger et Hamilton (1951)*	*Nombre de cas*	*Survie ⩾ 13 j.*	*Arcs anormaux*
3000 r.	16 à 18	11	7	6
	19 à 21	14	11	8
4000 r.	16 à 18	21	2	2
	19 à 21	8	3	3

Pour une même dose de rayonnement le pourcentage de survie est plus grand que lors de l'irradiation totale du cœur.

Effet de cette irradiation sur le système artériel

Le pourcentage d'anomalies vasculaires pour une dose de 3000 r. est sensiblement le même que lors de l'irradiation totale, c'est-à-dire environ 80 pour cent des cas. Les anomalies obtenues se répartissent de la façon suivante:

Anomalies portant sur des arcs subsistant normalement:

Arc 4 droit	absent	5 cas
	donnant la carotide	4 cas
Troncs brachiocéphaliques aberrants		14 cas
,, ,,	absents	1 cas
Arc 6 droit de diamètre réduit		4 cas

Persistances de vaisseaux devant normalement disparaître:

Racines aortiques	à droite	4 cas
	à gauche	2 cas
	bilatéralement	1 cas
Arc 4 gauche		1 cas
Artères sous-clavières primaires		9 cas

La région bulbo-troncale semble donc être responsable des malformations vasculaires produites par l'irradiation du cœur.

Malformations cardiaques

Sur les 23 cœurs étudiés, 3 seulement étaient normaux. Les 20 autres présentaient des défectuosités de la cloison aortico-pulmonaire et des valvules semi-lunaires.

Anomalies de la cloison aortico-pulmonaire

Elles s'observent aux deux étages bulbaire et troncal et présentent 3 degrés d'importance.

Tronc normalement cloisonné et communication entre les rampes au niveau du bulbe. Dans ce cas les crêtes bulbaires sont ébauchées, mais au niveau de la portion proximale du bulbe elles ne se soudent pas et laissent ainsi subsister une communication entre les rampes aortique et pulmonaire sous la forme d'une fissure plus ou moins importante.

Tronc cloisonné et bulbe indivis. Les crêtes bulbaires sont alors totalement supprimées et les courants sanguins des deux ventricules se mélangent complètement.

Absence complète de la cloison aortico-pulmonaire. Le bulbe est indivis et se prolonge à la sortie du cœur par un tronc artériel unique qui se ramifie plus loin selon une disposition plus ou moins proche de la normale (Planche, fig. B).

Anomalies vasculaires

La suppression ou l'inachèvement de la cloison bulbo-troncale s'accompagnent de malformations des valvules semi-lunaires.

Lorsque le tronc artériel est normalement cloisonné, tandis que le septum bulbaire est incomplet ou absent, on trouve fréquemment des valvules semi-lunaires de taille réduite. Lorsque la cloison bulbo-troncale fait totalement défaut, trois cas peuvent se présenter.

1. Les valvules semi-lunaires sont au nombre de 4. Stéphan (1952) a observé cette disposition en produisant la suppression de la cloison bulbo-troncale par les ligatures d'arcs aortiques.

2. Il existe seulement 3 valvules semi-lunaires, dont la situation suggère qu'elles appartiennent à l'une des rampes artérielles, celles de l'autre rampe étant absentes.

3. Enfin, on peut observer une absence complète de valvules. Il existe alors des bourrelets plus ou moins importants en relief dans la lumière du tronc artériel.

En conclusion, l'irradiation localisée du bulbe et du tronc permet d'obtenir isolément les anomalies observées au niveau de ces formations lors de l'irradiation de la totalité du cœur. D'autre part, les défectuosités de la cloison bulbo-troncale ainsi réalisées ne s'accompagnent pas de la persistance du foramen inter-ventriculaire. La cloison inter-ventriculaire et le départ de la rampe aortique sont normalement constitués.

Irradiation du ventricule (*Planche, fig. C*)

La limite antérieure de l'irradiation est donnée par le sillon ventriculo-bulbaire visible sur la face externe du cœur.

92 embryons ont reçu 3000 r. aux stades 16 à 18. Vingt-quatre ont survécu plus de 13 jours et ont pu être étudiés.

On retrouve dans beaucoup de cas certaines anomalies qui sont observées lors de l'irradiation totale du cœur, telles que l'hypertrophie ventriculaire gauche ou la présence d'un sillon plus ou moins profond séparant les apex ventriculaires.

Les malformations internes concernent la connection du ventricule gauche avec le bulbe, c'est-à-dire la naissance de la rampe aortique.

Foramen inter-ventriculaire persistant (*7 cas*)

Cette anomalie traduit une perturbation de l'évolution normale du coussinet endocardique médian du canal atrio-ventriculaire. Normalement en effet, le foramen inter-ventriculaire est transformé en un canal connectant le ventricule gauche et la rampe aortique bulbaire par une prolifération du matériel endocardique. Cette persistance du foramen inter-ventriculaire n'empêche pas la formation de la cloison bulbo-troncale qui était normalement constituée dans tous les cas étudiés. De même, le reste du septum inter-ventriculaire s'est édifié normalement.

Anomalie de la muscularisation de la rampe aortique (*14 cas*)

La base de la rampe aortique forme une volumineuse poche à paroi mince faisant parfois hernie dans l'oreillette gauche.

Une coupe transversale du cœur au niveau des canaux atrio-ventriculaires montre que la cloison inter-ventriculaire est très mince, peu muscularisée, ce qui permet cette dilatation considérable de la rampe aortique (Planche, fig. D).

Ici encore, la malformation résulte d'une évolution anormale du matériel endocardique atrio-ventriculaire.

Les cœurs ayant été irradiés en totalité n'ont pas présenté cette malformation isolément, mais possédaient souvent un bulbe indivis extrêmement vaste, occupant une position médiane. Il s'agissait alors de l'association de deux perturbations différentes de l'organogenèse normale. D'une part la cloison aortico-pulmonaire ne s'était pas formée, et d'autre part la déficience de la muscularisation de la partie crâniale du septum inter-ventriculaire s'était accompagnée d'une dilatation de la base de la rampe aortique.

En conclusion, l'irradiation de la partie de l'ébauche cardiaque située en arrière du sillon ventriculo-bulbaire provoque une perturbation de la connection du ventricule gauche et du bulbe ainsi que de la muscularisation de la cloison séparant les canaux atrio-ventriculaires droit et gauche.

Irradiation du sommet de la courbe ventriculaire (*Fig. 1a*)

Les expériences précédentes, de même que l'irradiation totale du cœur, n'empêchent pas le développement des trabécules myocardiques qui, en progressant de l'apex vers le canal atrio-ventriculaire, édifient le septum inter-ventriculaire musculaire. On pouvait donc se demander si une dose de rayonnement beaucoup plus forte, localisée au sommet de la courbe ventriculaire, aurait un effet sur l'évolution des trabécules myocardiques.

Dix-neuf embryons, aux stades 16 à 18, ont reçu 4900 r. Quinze d'entre eux ont survécu au-delà du 13ème jour de l'incubation. La survie est donc très bonne

malgré l'importance de la dose de rayonnement. Mais aucune malformation n'a été observée. Il est donc possible d'envisager que les lésions peuvent être réparées lorsque le champ de l'irradiation est très réduit.

Irradiation d'une zone ventriculo-bulbaire (*Fig. 1b*)

Dix-huit embryons, aux stades 16 à 18, ont reçu 3000 r. La mortalité est également faible pour cette série expérimentale, puisque 12 embryons ont survécu au-delà du 13ème jour de l'incubation.

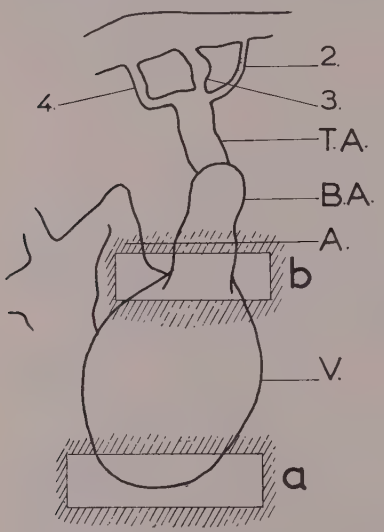

FIG. 1. Schémas des irradiations: *a*, du sommet de la courbe ventriculaire; *b*, de la zone ventriculo-bulbaire; 2., 3., 4., 2ème, 3ème, 4ème arcs aortiques; T.A., tronc artériel; B.A., bulbe artériel., A., atrium, V., ventricule.

Aucune malformation n'a été constatée au niveau des rampes artérielles, la cloison aortico-pulmonaire s'est édifiée normalement dans tous les cas. Une même dose de rayonnement, appliquée à l'ensemble de l'ébauche bulbo-troncale produit cependant un important pourcentage d'anomalies du cloisonnement.

Il apparaît donc que des doses qui provoquent d'importantes malformations lorsque la zone irradiée est étendue, ne sont pas capables de perturber l'organogénèse cardiaque lorsque le champ de l'irradiation est réduit.

CONCLUSIONS

L'irradiation aux rayons X de différentes parties de l'ébauche cardiaque de l'embryon de Poulet au 3ème jour de l'incubation a été réalisée pour étudier le mécanisme de l'apparition des malformations cardio-vasculaires obtenues par l'irradiation de l'ensemble du cœur.

1. La région bulbo-troncale semble être responsable des malformations vasculaires qui sont un effet indirect de l'irradiation du cœur.

2. L'irradiation de l'ébauche bulbo-troncale provoque des malformations de la cloison aortico-pulmonaire et des valvules semi-lunaires.

3. L'irradiation du ventricule primitif perturbe la connection de la rampe aortique avec le ventricule gauche et empêche la muscularisation normale de la cloison séparant les canaux atrio-ventriculaires. Il en résulte une dilatation de la base de la rampe aortique.

4. Lorsque le champ de l'irradiation est très petit, l'organogénèse cardiaque n'est pas perturbée, même par des doses fortes. Il semble donc que dans ces conditions l'ébauche cardiaque possède un pouvoir de réparation des lésions produites par le rayonnement dans un petit territoire du cœur.

5. Enfin, certaines parties du cloisonnement cardiaque n'ont jamais été affectées par l'irradiation du cœur. En particulier, le septum interventriculaire musculaire s'est toujours édifié normalement à partir des trabécules myocardiques. Toutes les parties de l'ébauche cardiaque n'ont donc pas la même sensibilité, ce sont les dérivés du matériel endocardique qui sont le plus souvent perturbés.

SUMMARY

Different parts of the heart rudiment of the chick embryo at 3 days of incubation were irradiated with X-rays to study the mechanism of production of the cardio-vascular anomalies obtained by irradiating the whole heart.

(1) The bulbo-truncal region seems to be responsible for the malformations of the vessels, which are an indirect effect of the irradiation of the heart.

(2) Irradiation of the bulbo-truncal rudiment causes malformation of the pulmo-aortic septum and of the semilunar valves.

(3) Irradiation of the primitive ventricle disturbs the connexion of the aortic trunk with the left ventricle and prevents the normal muscularization of the partition separating the atrio-ventricular canals. There results a dilatation of the base of the aortic trunk.

(4) When the field irradiated is very small, cardiac morphogenesis is not disturbed even by high doses. It seems, therefore, that in these conditions the heart rudiment has the power of repairing lesions produced by irradiation of a small region.

(5) Finally, some of the partitioning of the heart was never affected by irradiation of the heart. In particular, the muscular interventricular septum is always normally formed from the myocardial trabeculae. All parts of the cardiac rudiment are not equally sensitive; it is the derivatives of the endocardial material which are most often disturbed.

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EXPLICATION DE LA PLANCHE

FIG. A. Schéma de l'irradiation du bulbe et du tronc artériel. 2., 3., 4., 2ème, 3ème, 4ème arcs aortiques; T.A., tronc artériel; B.A., bulbe artériel; A., atrium; V., ventricule.

FIG. B. Coupe transversale du cœur d'un embryon ayant subi l'irradiation du bulbe et du tronc artériel. La base du tronc (T.I.) est indivise, le tronc se ramifie ensuite en vaisseaux aortique (A.) et pulmonaire (P.).

FIG. C. Schéma de l'irradiation du ventricule. (Même légende que pour la fig. A.)

FIG. D. Coupe transversale du cœur d'un embryon ayant subi l'irradiation du ventricule. La rampe pulmonaire (R.P.) est normale, la rampe aortique (R.A.) forme une poche dilatée.

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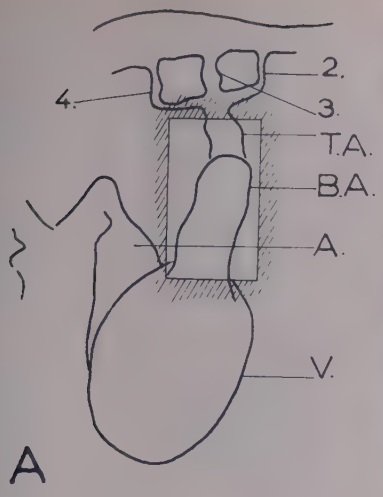
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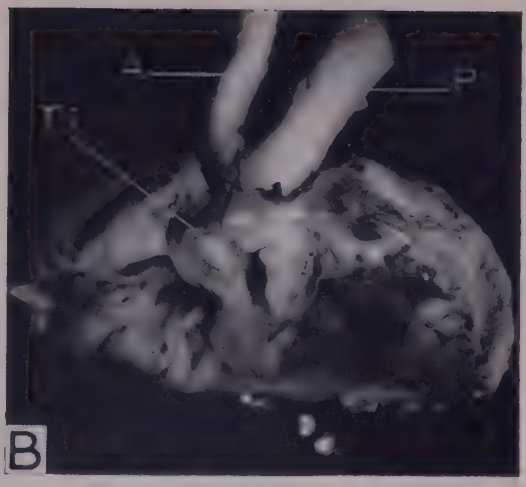
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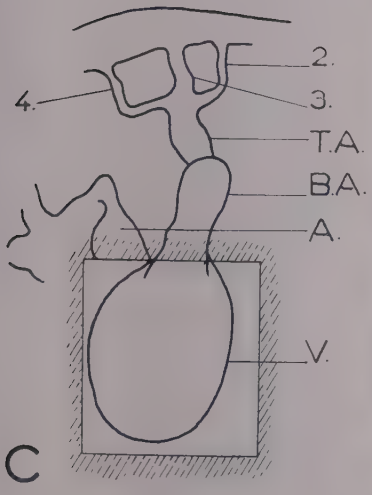
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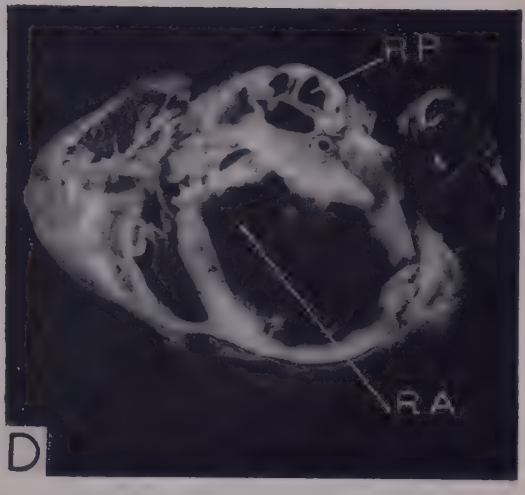
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Serologically Active Groups of Amphibian Embryos

by KAZUKO INOUE[1]

From the Department of Pathology, School of Medicine, University of Kanazawa, and Kanazawa Women's College, Japan

INTRODUCTION

CHANGES in antigenic composition during embryonic development of the vertebrates have been reported for a large number of species, as has been reviewed by Tyler (1955), Woerdeman (1955), Nace (1955), and others. In particular Cooper (1948), Flickinger & Nace (1952), and Spar (1953) have considered such changes in anuran development, while Clayton (1953), Inoue (1951, 1952, 1954, 1961 *a*, *b*), and Nace & Inoue (1957) tested their anti-amphibian sera in whole embryos and tissue cultures to determine whether or not morphogenetic events would be modified by such exposure. The data obtained from these studies and from works on other organisms indicates that combination of an antibody with its appropriate antigen *in vivo* leads to specific modification of morphogenesis or to cytolysis. Thus, Inoue (1961 *a*) reports that antisera directed against gastrula supernatant preparations specifically inhibited the development of the gastrula, and that anti-neurula supernatant specifically inhibited the development of the neurula.

It has been assumed that the antigenic materials responsible for production of these antisera are high molecular weight substances. The possibility has not been excluded from consideration that, though antigenic changes do occur, these are not changes in the population of macro-molecules but simply of the surface groupings which they present to the antibody producing mechanism. Landsteiner (1945) has discussed the serological behaviour of antigens following oxidation, reduction, acylation, and other treatment. On this basis he concluded that relatively circumscribed reactive groups located on the surface of the protein or other antigenic components are more important in determining serological specificity than the nature of the molecular structure as a whole. Thus, it would seem necessary to examine the possibility that such changes in the antigens occur during embryogeny.

An approach to this problem was suggested by the reviews of Herriott (1947) and Olcott & Fraenkel-Conrat (1947) on the determination of the specific surface groups of protein molecules. The methodology employed consists of treatment of purified 'simple' proteins with various reagents selected for their

[1] *Author's address:* Department of Pathology, School of Medicine, University of Kanazawa, Kanazawa, Japan.

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ability to combine with specific radicals. Measurement of the uptake of these reagents provides an index of the number of such radicals which are exposed.

Although the supernatants prepared by homogenization of embryos of various stages in phosphate buffer (Inoue, 1961 *a*) were complex antigenic mixtures, it seemed possible that treatment with the selected group-reaction reagents would block specific active groups or groupings, thereby modifying the appropriate precipitin reactions. Limitations of the precipitin method as a quantitative measure renders impossible an absolute determination of the number of specific reactive sites, but it could provide an estimate of the significance of their participation in embryogenesis at a given stage and could therefore be expected to provide data on the stereochemical configurations concerned in growth and differentiation.

This report deals with (1) the number and distribution of specific reactive groups on, and (2) the serological modifications of antigens in the phosphate buffer soluble preparations of developmental stages of the urodele *Triturus pyrrhogaster*. The determination of these groups and the production of the serological modification was accomplished by treatment with nine reagents chosen for their ability to combine with known specific groups on protein molecules, and for their applicability under conditions minimizing denaturation.

Specific reactive groups are shown to characterize specific embryonic stages, and an effort is made to correlate their chemical activity with concomitant morphogenetic events.

MATERIALS AND METHODS

Embryonic materials

S-fraction antigens

Following the procedures more extensively described by Inoue (1961 *a*), *T. pyrrhogaster* embryos were collected, staged, and homogenized in M/15 Sørensen's phosphate buffer (pH 7·2 to 7·4). These homogenates were centrifuged at 1,600 g. for 20 minutes in the cold condition and the supernatants collected, yielding a solution of water-soluble materials including nucleo-, muco-, and lipo-proteins. Supernatants to the following stages were prepared and are designated throughout this report by the letters shown in parentheses with the stage designation: large oocyte (LOS, collected from oviduct and uterus), morula (MS, stage 7, according to Okada & Ichikawa, 1947), blastula (BS, stage 10), early gastrula (G_1S, stage 12), late gastrula (G_2S, stage 14), neurula (NS, stage 18), and tail-bud (TBS, stage 27).

V-fraction antigens

The residue from the above centrifugations was washed ten times with phosphate buffer, dissolved in 10 per cent. NaCl, stored overnight at 2° C., and centrifuged at 1,600 g. for 20 minutes. The supernatant contained the

components of the solvated yolk granules and is hereafter designated the *V*-fraction.

Anlage antigens

For regional studies of the embryo the animal and vegetal halves of the blastula were separated by cutting through the marginal zone; and embryos of the gastrula and neurula stages were divided into dorsal and ventral regions. The dorsal region was composed approximately of presumptive areas for the nervous system, notochord, somites, and the anterior half of the alimentary tract in the gastrula, and of the derivatives of these areas in the neurula stages. The remainder of the embryo was designated ventral in both cases.

Preparation of Antisera

Each *S*- and *V*-fraction prepared from embryos of the above stages was injected into at least two young white rabbits weighing approximately 2 kg. Inoculation was made through the posterior marginal ear vein according to the following schedule: first week, 100 embryos injected in 3 aliquots on alternate days; second week, rest period; third week, as first week with 150 to 200 embryos; fourth week, rest period; fifth week, as first week with 200 to 300 embryos. Following the initial injection, desensitizing injections were made intraperitoneally one hour before each intravenous injection. The antibody titres were tested on small samples collected periodically, and when the titre was sufficiently high, usually round about the seventh week, the rabbits were exsanguinated from the carotid artery. Control sera were obtained from three uninjected rabbits.

All sera were collected under sterile conditions and were not Seitz filtered or treated with antibiotics. Complement was destroyed by heating at 56° C. for 30 minutes and the sera were stored at 4° C. until the time of use. The antisera are designated throughout this paper by the prefix 'anti-' which is followed by the designations given above for the *S*- and *V*-fractions.

Precipitin reactions of these and similar sera before and after absorption with a number of embryonic reagents have been reported by Inoue (1961 *a*).

Method for precipitation test

A micro-precipitin test was developed in which 0·02 ml. of 2^n diluted test antigen (standard solution is 0·1 ml. per embryo) was added to 0·1 ml. of antiserum in a depression slide, stirred, allowed to stand for 5 hours at 15° C., and finally placed at 4° C. for 1 hour. The end-point titres were determined by microscopic examinations; in some cases the dark field technique was used.

Group reaction reagents and their use

The method for quantitative analysis of reactive groups on protein molecules have not been perfected because very few reagents have demonstrated sufficient group specificity. However, those reagents whose reactions have been charac-

terized may be used for selective blocking of certain reactive groups which may be of antigenic significance. The reagents chosen and the groups with which they react are given in Table 1. This table was prepared from data collected by Olcott & Fraenkel-Conrat (1947) and Satake (1949). The symbols used in the table follow the designations indicated by these reviewers.

TABLE 1

*Effects of reagents on protein groups.**

Groups: / *Reagents*	*Sulphydryl* —SH	*Disulphide* —S—S—	*Phenol* —OH	*Indol*	*Imidazol*	*Amino* $-NH_2$
p-Chloromercuribenzoate (PCMB)	3+	—	—	—	—	—
Monoiodoacetamide (MIA)	2+	—	±	—	—	±
Cysteine (R—SH)	—	3+	—	—	—	—
Iodine (I) . .	3+	—	3+	+?	±	—
Tyrosinase (T-ase).	—	—	2+	—	—	—
2,4-Dinitrofluorobenzene (DNFB)	3+	—	3+		±	3+
Phenyl isocyanate (PIC) . .	3+	—				3+
Benzoyl chloride (BC) . .		—	±			3+
Diazonium compounds (DC) .		—	2+	±	3+	

* The symbols used have the following significance: 3+, 2+, and + indicate relative rapidity or extent of reaction, with 3+ denoting the most rapid reaction. ± indicates reactions that may or may not occur under the conditions suggested. — indicates those reactions that either have been shown not to occur or that appear improbable from organic chemical considerations. Spaces have been left blank where there is a possibility of reaction but where no evidence is available. (After Olcott *et al.* (1947) and Satake (1949).)

Because of the complex composition of the *S*-fractions of each embryonic stage, treatment with the various reagents was conducted, in so far as possible, under comparable conditions.

The following methods were used.

p-Chloromercuribenzoate (PCMB).[1] On the basis of the demonstration by Hellerman *et al.* (1943) of the high selectivity of PCMB for sulphydryl (SH) groups of urease, this reagent was chosen as the most useful blocking reagent for SH.

The titrimetric method employed by MacDonnell *et al.* (1951) on ovalbumin was used to determine the quantity of PCMB necessary for reaction with the *S*- and *V*-fractions of each embryonic stage. This quantity of PCMB was added

[1] Initial quantities of PCMB were synthesized in our laboratory, subsequently it was obtained from Wako Pure Chemical Industries Ltd., Japan. The other reagents were obtained from either Merck (Germany, U.S.A.) or Wako Ltd. (Japan)).

dropwise under continuous stirring to *S*-fractions adjusted to pH 7·0 with N 10 HCl. The reaction was allowed to continue for 30 minutes at room temperature (about 15° C.) after which unreacted PCMB was removed by dialysis.

Monoiodoacetamide (MIA). Although iodoacetate or iodoacetamide has been used to treat many substrates in the classification of enzymes attacking their SH groups, alkylation with MIA lacks the desired specificity for SH groups. This reagent has, however, been utilized in this study in support of data obtained with PCMB.

One-tenth ml. of purified 0·05 M MIA solution per embryo was added to the *S*-fractions which had been brought to pH 7·2 with phosphate buffer. The reaction was allowed to proceed for 2 hours at 5° C. Unreacted reagent was removed by dialysis.

Cysteine (R-SH). Cysteine is a well-known reducing agent for disulphide linkages (—S—S—) in the protein molecule (Barron *et al.*, 1947).

One-tenth ml. of 0·1 M recrystallized cysteine per embryo was added to each *S*-fraction. The pH was adjusted to 7·4 with phosphate buffer, and the reaction was allowed to proceed for 2 hours at 5° C. Again excess reagent and denatured protein were removed by dialysis and centrifugation respectively.

Iodine (I). The action of iodine on protein molecules is oxidation of SH groups, iodination of phenol groups, and a slight inactivation of indol and imidazol groups. The nature of the reaction depends upon conditions such as pH, as noted in Herriott's (1947) review of work done by Reineke & Turner (1945). Iodination has been utilized, for example, by Herriott (1937) to determine the tyrosine content and activity of pepsin, while Harington & Neuberger (1936) used it for the determination of tyrosine in the insulin molecule.

A standard solution of 0·003 N iodine was prepared and titrated with the supernatant in accordance with the Folin & Ciocalteu (1927) colour reaction or the starch colour test for excess iodine. On the basis of such preliminary determinations, approximately 0·02 ml. of this iodine solution per embryo was added to each *S*-fraction, adjusted to pH 8·0 with N 10 NaOH, and allowed to react in an ice bath for 2 hours. Excess iodine was removed by dialysis, although subsequently it was found that sodium thiosulphate solution could be used to inactivate the excess iodine without modification of the precipitin reactions. Following iodination, pH was readjusted to 7·4 with N 10 HCl.

Tyrosinase (T-ase). Although the specificity of tyrosinase is somewhat dubious, Sizer (1946) has presented data which indicate the ability of this enzyme to oxidize some of the tyrosine (phenol) groups on certain proteins. Therefore, it seemed feasible to utilize tyrosinase to inactivate phenol groups and thus provide information auxilliary to the data obtained by iodination.

Because of the difficulty of obtaining purified tyrosinase, homogenate of potatoes was used. This solution was filtered and dialysed against distilled water for 5 days at 4° C., and it was then centrifuged. Equal volumes of enzyme

solution and antigen were combined and shaken at room temperature for 2 hours. After centrifugation the supernatant was used as a test antigen. Due to the difficulty of removal of the excess tyrosinase, there may have been some action of the enzyme on the antibody molecules. As will be seen, the results suggest only comparative stage specificity.

Dinitrofluorobenzene (*DNFB*). Using 2,4-dinitrofluorobenzene, Sanger (1945) determined the number and nature of free amino groups on insulin; subsequently, the ability of DNFB to bind free SH and phenol as well as amino groups was demonstrated. With the possible exception of imidazole, other groups are little affected by this reagent (Herriott, 1947).

Embryonic supernatant preparations were saturated with DNFB and allowed to react with agitation at room temperature at pH 7·4 for 2 hours and then at 0° C. overnight. Subsequent to this treatment the solution showed a light yellow colour caused by the reaction of DNFB with the —SH and —NH_2 groups of the protein. After dialysis and centrifugation, the supernatant was used as test antigen.

Phenylisocyanate (*PIC*) *and benzoyl chloride* (*BC*). In a manner similar to that of many of the other reagents described above, acylation with isocyanate results in inactivation of SH and NH_2 groups, as has been demonstrated by Hopkins & Wormall (1934) in their studies on insulin. In the present case, embryonic supernatants were saturated with PIC and left to react at 0° C. for 2 hours; during this reaction the pH was maintained at 8·0 with N/10 NaOH.

Benzoylation, i.e. the Schotten–Baumann reaction, is known to inactivate the amino groups of proteins. There are reports of its activity with phenol, indol, imidazol, and sulphydryl groups (Olcott & Fraenkel-Conrat, 1947); however, the validity of these is doubtful.

Five-hundredths ml. of BC per embryo was added to the *S*-fraction; the reaction was allowed to continue at pH 8·0 for 2 hours and was followed by dialysis and centrifugation.

Both of these reagents caused extensive denaturation of the proteins in the supernatant preparations. Therefore, results obtained with them should be viewed with caution and utilized only as supplementary data.

Diazonium Compound (*DC*). Azoprotein compounds have been used as haptens by immunologists for some time, as has been detailed in the reviews of Landsteiner (1945) and Boyd (1956). They have also been used in the determination of phenol and imidazol groups on enzymes, hormones, and fibroin as reviewed by Herriott (1947) and Satake (1949). Thus, the possibility of utilizing diazo-coupling for the inactivation of phenol and imidazol groups on the protein molecule is suggested.

The salt of either diazo-sulphanylic acid or diazo-arsenylic acid was used in this reaction. To determine the reaction volumes, a series of embryonic fractions were treated with volumes of DC standard solution (0·013 M) ranging from 0·020 ml. to 0·10 ml., at intervals of 0·001 ml., per ml. of fraction. These were

adjusted to pH 8·0 and agitated at 0° C. for 30 minutes. The amount of DC remaining after this diazo coupling was tested with Toyo No. 2 filter paper saturated with a solution of β-naphthol in 100 per cent. alcohol and was quantitated by comparison with a standard colour series. This preliminary chemical analysis was utilized in calculation of the minimum amount of DC required for inactivation of these groups. The amounts thus determined were added to the *S*-fraction, the pH adjusted to 8·0, and the reaction allowed to proceed at 0° C. for 1 hour. The pH was then corrected to 7·0, and the supernatant was used as antigen in the precipitation tests.

Tests

The protocol of control and experimental serological reactions were as follows: (1) untreated antigen tested against normal rabbit serum, (2) untreated antigen tested against antiserum, (3) treated antigen tested against normal rabbit serum, and (4) treated antigen tested against antiserum.

All treatments were identical except for the presence of the reagent. Stage differences constituted internal controls. Particular attention was paid to adjusting the pH of experimental and control tests to the same value. Thus, if the pH of experimental antigens was adjusted during the process of treatment with the reactive agents, the pH of control antigens was similarly adjusted. In all cases pH was finally adjusted to 7·0 to 7·4 prior to conducting the precipitin reactions.

Similar precautions were taken with regard to the dilution of antigens during the process of treatment.

RESULTS

Tests with each reagent were repeated at least 15 times per rabbit, and the mean values were recorded as the result for each series.

TABLE 2

Micro-precipitin titres of control tests

(Values in n of antigen dilution, 2^n)

Antigen: / *Antisera*	*LOS*	*MS*	*BS*	*G_1S*	*G_2S*	*NS*	*TBS*
Anti-LOS .	13	11	11	11	10	10	10
Anti-MS .	12	14	13	12	12	12	12
Anti-G_1S .	10	12	14	15	14	13	12
Anti-G_2S .	10	12	13	14	15	14	13
Anti-NS .	10	12	13	13	13	15	14
Anti-TBS .	10	12	12	12	13	13	14

See pp. 564 and 565 for explanation of abbreviations.

Controls

Micro-precipitin test end point titres obtained with untreated antigens are listed in Table 2. The figures represent the values of n in the antigen dilution

series 2^n. All antisera had high titres. In each case the homologous titre was the highest and all reacted with every antigen preparation regardless of stage.

No precipitation was observed with treated or untreated antigen tested against normal rabbit serum.

No difference in reaction could be attributed to the alteration of pH of the antigens in the process of treatment, as in all cases the titres of control antigens, the pH of which had been modified to conform to the pH changes experienced by the experimental antigens, were identical with the titre of antigens whose pH had not been so modified.

Experimentals

The degree of inhibition of precipitation obtained with antigens treated with the group reaction reagents was expressed as the difference between the experimental titres and the control values shown in Table 2. It is assumed that these differences in titre are proportional to the degree of specific inactivation of the reactive groups shown in Table 1, and to the degree of their participation in the serological reaction.

Results of PCMB treatment

Titratable SH *groups*. Biochemical analyses utilizing *p*-chloromercuribenzoate for quantitation of SH groups on macromolecules derived from various developmental stages are summarized in Table 3. The values represent averages of the amount of PCMB in μM per embryo required to titrate the SH groups and were calculated from more than eight determinations per stage and were accurate to two places. Table 3 A shows these values obtained with the *S*-fractions and *V*-fractions of whole embryos. The *S*-fraction of small oocytes showed a steady increase in the value during oogenesis and cleavage and reached a peak at the morula stage, and then gradually decreased during further development. The *V*-fraction, however, maintained the initial low value during oogenesis. After fertilization a maximum value was attained which was maintained until the early gastrula and then followed by lower values.

The distribution of SH groups in various regions of the embryo is indicated in Table 3 B. The soluble components of the animal half of the blastula were richer in PCMB titratable SH groups than was the vegetal half. However, there was little evidence of a dorso-ventral gradient in the gastrula. A marked gradient again appeared in the neurula stage. The distribution of titratable SH groups observed by this method bears a striking similarity to that found by cytochemical determinations (Inoue, 1951) of cystine-cysteine (SS–SH) compounds in *Triturus* embryos. Regional distribution of titratable SH groups was scarcely perceptible in the *V*-fraction at any stage.

Inhibition of precipitation by PCMB treatment. Precipitation tests were conducted on *S*-fractions the SH groups of which had been blocked by use of

PCMB in amounts determined by previous titration (cf. Table 3). Antigenic preparations thus treated produced considerable reductions in precipitin titres

TABLE 3

Titration of SH *groups on S- and V-fractions from* Triturus *embryos with* p-*chloromercuribenzoate* (*PCMB*)

A. μM of bound PCMB per embryo

Stage	*S-fraction*	*V-fraction*
Small oocyte	26·0±0·5	36·0±0·5
Large oocyte	47·0±1·0	36·0±0·5
2-cell stage	78·0±1·0	73·0±1·0
Morula	89·0±0·5	78·0±0·5
Blastula	84·0±0·5	78·0±0·5
Early gastrula	57·0±0·0	78·0±0·5
Late gastrula	52·0±0·5	57·0±0·5
Neurula	47·0±0·5	47·0±0·5
Tail-bud stage	42·0±0·5	42·0±0·5

B. μM of bound PCMB per embryo

Stage	*Region*	*S-fraction*	*V-fraction*
Blastula	Animal half	58·0±1·0	39·0±0·5
	Vegetal half	26·0±0·5	39·0±0·5
Gastrula	Dorsal half	31·0±0·5	36·0±0·5
	Ventral half	26·0±0·5	42·0±0·5
Neurula	Dorsal half	31·0±0·5	21·0±0·5
	Ventral half	16·0±0·5	26·0±0·5

compared with the control values listed in Table 2. Table 4 gives the reduction in titres, using the average values from repeated tests as n of the $2^n = (2^a - 2^b)$, where 2^a is the value for the unmodified antigen and 2^b is the value for the treated antigen. As suggested above, this titre reduction value is assumed to bear a significant relationship to the extent of participation of SH in the immunological integrity of the antigenic molecules involved.

TABLE 4

Inhibitory effects on precipitation tests of S-fractions by treatment with p-*chloromercuribenzoate* (*PCMB*)

(See text for abbreviations)

Treated antigen: / *Antisera*	*LOS*	*MS*	*BS*	G_1S	G_2S	*NS*	*TBS*
Anti-LOS	5·5	6·0	6·0	5·0	4·0	1·0	1·0
Anti-MS	6·0	8·5	9·2	6·7	6·0	3·7	3·7
Anti-G_1S	2·0	6·7	7·0	6·5	5·7	4·7	3·7
Anti-G_2S	2·0	5·7	6·0	6·0	5·7	5·3	4·3
Anti-NS	2·0	7·0	6·0	5·0	5·0	5·0	4·0
Anti-TBS	1·0	4·0	4·0	4·0	4·0	4·0	4·0

It is clear that the strongest inhibitions of serological activity were exhibited by the PCMB-treated MS and BS either against homologous or heterologous antisera. Among the various antisera, titres with anti-MS were most sensitive to PCMB treatment of *S*-fractions. Of all the combinations tested, the PCMB treated BS against anti-MS system gave the greatest titre reduction, i.e. $(2^a - 2^b) = 2^{9 \cdot 2}$. The peak effects were seen with MS and BS and tended to fall off in later stages of development, as did the PCMB titration values.

The data suggest either that the operations involved in PCMB treatment of the *S*-fraction lead to drastic but unidentified serological modifications of the antigens, or that SH groups play a significant role in maintaining the serological specificity of these antigens of the embryo. The titration and precipitin data are in accord, as can be seen by a comparison of the data of Tables 3 and 4. As protein concentrations were similar, the major variable among these tests was the difference in antigenic composition from stage to stage. Thus, it would seem that these data support the suggestion that the serological results are indeed a reflection of the serological significance of the SH groups rather than of a non-specific denaturation resulting from the procedural aspects of the treatment.

Results of MIA treatment

Although monoiodoacetamide binds SH groups, the influences of monoiodoacetamide treatment on the serological activity of *S*-fraction are shown in Table 5 utilizing the same conventions as for Table 4. In general, the results with MIA were lower than those with PCMB. But again, MS and BS were strongly affected, anti-MS being most sensitive to the modifications. The influence of the MIA was less in the later stages. The conclusions suggested by PCMB treatment were thus supported by these data.

TABLE 5

Inhibitory effects on precipitation tests of S-fractions by treatment with monoiodoacetamide (MIA)

(See text for abbreviations)

Treated antigen:						
Antisera	*MS*	*BS*	*G_1S*	*G_2S*	*NS*	*TBS*
Anti-MS . .	6·0	6·5	4·0	3·5	3·0	3·0
Anti-G_1S . .	4·0	6·0	6·0	5·0	3·5	2·5
Anti-G_2S . .	3·5	4·0	5·0	4·5	4·0	3·0
Anti-NS . .	2·0	3·0	3·0	3·0	3·0	3·0

Results of R—SH *treatment*

Inhibition of precipitin titres by cysteine treatment of *S*-fractions are listed in Table 6. Cysteine (R—SH) blocks disulphide groups. Thus increased inhibition of precipitin titres suggests an increased number of SS groups related to

antigen-antibody reactions. On this basis the present data testify to an increase in activity of SS between large oocyte and morula, a significant decrease between morula and blastula, and a second rise at the early gastrula stage, followed by a gradual decrease through subsequent stages. Antisera directed against all stages yielded essentially parallel modifications of titres suggesting that if SS groups are of serological significance their influence is less with regard to stimulation of antibody production than with their influence on the test reaction of precipitation.

TABLE 6

Inhibitory effects on precipitation tests of S-fractions by treatment with cysteine (R—SH)

(See text for abbreviations)

Treated antigen: / *Antisera*	*LOS*	*MS*	*BS*	G_1S	G_2S	*NS*	*TBS*
Anti-LOS . .	6·0	6·0	4·0	5·0	4·0	4·0	3·0
Anti-MS . .	6·0	8·0	5·0	6·0	6·0	6·0	5·0
Anti-G_1S . .	4·0	7·0	6·5	9·0	8·5	7·0	5·0
Anti-G_2S . .	2·0	7·0	6·0	9·0	8·5	8·0	6·0
Anti-NS . .	2·0	7·0	6·0	7·0	6·0	6·0	6·0
Anti-TBS . .	2·0	7·0	5·0	6·0	6·0	6·0	6·0

Comparison of the PCMB and MIA data indicates a reciprocal relationship between —SH and —S—S— groups, particularly at the blastula and gastrula stages, an observation which suggests that —SH ⇌ —S—S— transformations may be important aspects of the events occurring at these stages.

Results of I-treatment

Sulphydryl and phenol groups are blocked by iodination. Table 7 shows the inhibition of precipitin titres produced by this reaction, and, following the reasoning utilized above, it also indicates the stereochemical activity of these groups in the serological reaction.

TABLE 7

Inhibitory effects on precipitation tests of S-fractions by treatment with iodine (I)

(See text for abbreviations)

Treated antigen: / *Antisera*	*LOS*	*MS*	*BS*	G_1S	G_2S	*NS*	*TBS*
Anti-LOS . .	7·0	3·0	4·0	4·0	4·0	3·0	2·5
Anti-MS . .	6·0	5·0	5·0	5·5	6·0	5·0	4·5
Anti-G_1S . .	5·0	4·5	8·0	9·0	9·0	6·5	5·5
Anti-G_2S . .	5·0	4·5	7·2	9·2	9·2	8·0	6·7
Anti-NS . .	4·0	4·5	6·5	7·5	8·0	7·5	7·0
Anti-TBS . .	4·0	4·5	5·0	6·0	6·0	5·5	5·0

The character of the reactions of iodinated LOS differs from other data obtained following iodination. Iodinated LOS tested against anti-LOS gave an inhibitory value of 2^7, but it gave lower values with all other antisera. Also, anti-LOS yielded the lowest inhibition values of any antisera tested against any iodinated *S*-fractions. Thus, the conclusion may be drawn that the SH and the phenol-OH groups have a rather high activity in large oocytes but that they are related to antigens of different specificity than are these same groups at later stages, such as the functional groups for fertilization membrane formation. Since the SH groups demonstrated no unique activity at the large oocyte stage in the tests with PCMB, it might safely be concluded that in this case the phenol groups or a combination of SH and phenol groups were responsible for the activity observed in LOS.

The reactions most severely affected by iodination were those with G_1S or G_2S against anti-G_1S or anti-G_2S, in which the inhibitory values were more than 2^9. The curves of the other antisera followed a similar though less marked pattern, indicating that the peak of the inhibitory effects of iodination on precipitation is found at this stage of development.

Results of T-ase treatment

The inhibitory effects on precipitin reactions of *S*-fractions elicited by oxidation of phenol groups with tyrosinase were shown in Table 8. As in tests with other phenol blocking reagents, the serological influence of this group rose to a maximum in the reactions of GS against anti-GS, and then gradually declined.

TABLE 8

Inhibitory effects on precipitation tests of S-fractions by treatment with tyrosinase (T-ase)

(See text for abbreviations)

Treated antigen:						
Antisera	*MS*	*BS*	*G_1S*	*G_2S*	*NS*	*TBS*
Anti-MS . .	4·0	4·0	4·0	4·0	3·0	3·0
Anti-G_1S . .	4·0	6·0	7·0	7·0	5·0	4·0
Anti-G_2S . .	4·2	5·2	7·0	7·5	7·0	6·5
Anti-NS . .	4·0	5·0	5·0	5·5	5·0	5·0

Results of DNFB treatment

Table 9 indicates the degree of inhibition of precipitation due to treatment with 2,4-dinitrofluorobenzene, which reacts with the free amino groups of the lysine residues, the sulphydryl groups, the phenolic hydroxyl groups of the tyrosine residues, and some of imidazole groups of histidine residues. It is worth noting that the highest inhibitory values occurred with the antigen-antibody systems of the later gastrula, neurula, and tail-bud stage. This inhibition may be mainly due to the breakdown of amino groups by DNFB treatment,

because the foregoing tests involving only SH and phenol groups caused the greatest inhibitions in the earlier stages.

TABLE 9

Inhibitory effects on precipitation tests of S-fractions by treatment with 2,4-dinitrofluorobenzene (DNFB)

(See text for abbreviations)

Treated antigen: *Antisera*	*LOS*	*MS*	*BS*	*G_1S*	*G_2S*	*NS*	*TBS*
Anti-LOS . .	5·0	5·0	5·0	5·0	6·0	6·0	6·0
Anti-MS . .	4·0	4·5	4·5	4·7	4·7	5·2	5·2
Anti-G_1S . .	3·0	5·8	7·0	7·5	7·5	7·0	7·0
Anti-G_2S . .	3·0	6·0	7·0	8·0	8·0	9·0	8·5
Anti-NS . .	4·0	7·2	8·2	8·5	8·5	9·5	9·5
Anti-TBS . .	5·0	7·0	7·0	8·0	8·5	9·0	9·0

Results of PIC and BC treatment

The results of serological reactions with antigens treated with phenyl isocyanate are presented in Table 10. Further, Table 11 indicates the degree of

TABLE 10

Inhibitory effects on precipitation tests of S-fractions by treatment with phenyl isocyanate (PIC)

(See text for abbreviations)

Treated antigen: *Antisera*	*MS*	*BS*	*G_1S*	*G_2S*	*NS*	*TBS*
Anti-MS . .	4·0	4·0	3·0	3·0	3·0	3·0
Anti-G_1S . .	6·0	7·0	8·0	8·0	6·0	5·0
Anti-G_2S . .	6·0	7·0	8·0	8·0	8·0	7·0
Anti-NS . .	5·0	6·0	6·0	6·0	6·0	6·0

TABLE 11

Inhibitory effects on precipitation tests of S-fractions by treatment with benzoyl chloride (BC)

(See text for abbreviations)

Treated antigen: *Antisera* .	*MS*	*BS*	*G_1S*	*G_2S*	*NS*	*TBS*
Anti-MS . .	6·0	6·0	5·0	4·5	4·5	4·5
Anti-G_1S . .	5·5	7·0	7·5	7·5	7·0	6·0
Anti-G_2S . .	5·8	7·0	8·0	8·0	8·2	6·0
Anti-NS . .	6·0	6·5	7·2	8·0	8·0	7·0

inhibition of precipitation due to benzoylation with benzoyl chloride, which reacts with the NH_2 groups and possibly with the phenol groups. The results of

this series of tests do not contradict the foregoing suggestion that the maximum effects of inactivation of the amino groups may appear in the late gastrula and neurula stages.

Results of DC treatment

Titratable phenol, imidazol and indol groups. The titrations of *S*- and *V*-fractions of various developmental stages for groups which can be diazotized with diazosulphanilic acid are summarized in Table 12 on a per embryo basis. Determinations on *S*-fractions (Table 12 A) indicated a weaker combining capacity at a small oocyte stage with a gradual increase at the late gastrula,

TABLE 12

Titration of diazotizable groups on S- and V-fractions from Triturus *embryos*

A. μM of bound diazonium compound per embryo

Stage	*S-fraction*	*V-fraction*
Small oocyte .	49·0±0·5	14·0±0·5
Large oocyte .	49·0±0·5	14·0±0·5
2-cell stage .	55·0±1·0	14·0±0·5
Morula . .	55·0±0·5	17·0±0·0
Blastula . .	60·0±0·5	18·0±0·5
Early gastrula .	62·0±0·0	14·0±0·4
Late gastrula .	69·0±0·0	17·0±0·5
Neurula . .	64·0±0·5	18·0±0·0
Tail-bud stage .	59·0±0·5	17·0±0·5

B. μM of bound diazonium compound per embryonic region

Stage	*Region*	*S-fraction*	*V-fraction*
Blastula	Animal half	39·0±0·5	9·0±0·5
	Vegetal half	21·0±0·5	8·0±0·5
Gastrula	Dorsal half	35·0±0·5	8·0±0·5
	Ventral half	27·0±0·5	6·0±0·5
Neurula	Dorsal half	50·0±1·0	12·0±0·5
	Ventral half	14·0±1·0	6·0±0·5

followed by a slow decline. The values for *V*-fractions did not exceed 18 μM at any stage and were quite uniform throughout development. Inspection of Table 12 B indicates the presence of groups in the dorsal half of the neurula which were capable of diazotizing 50 μM of DC but only 14 μM of DC in the ventral half. Correspondingly, in the blastula and gastrula stage, the animal or dorsal half was found to be richer in diazotizable groups. Here, as in Table 3, data from Table 12 B were combined to obtain the appropriate value shown in Table 12 A.

Inhibition of the precipitin tests following DC treatment

DC treatment of the *S*-fraction inhibited precipitin reactions as shown in Table 13. Inhibitory effects were slight in reactions of DC-treated LOS against

each antiserum and in reactions of the DC-treated *S*-fraction of each stage against anti-LOS. The grade of inhibition rose with MS against each antiserum, and an increase of the value was seen in all the combinations of BS, G_1S, G_2S, and NS against anti-G_1S, anti-G_2S, and anti-NS.

TABLE 13

Inhibitory effects on precipitation tests of S-fractions by treatment with diazonium compound (DC)

(See text for abbreviations)

Treated antigen:							
Antisera	*LOS*	*MS*	*BS*	*G_1S*	*G_2S*	*NS*	*TBS*
Anti-LOS . .	3·0	3·0	4·0	4·0	4·0	4·0	4·0
Anti-MS . .	4·0	7·0	7·0	6·5	6·5	6·5	6·5
Anti-G_1S . .	3·0	7·5	9·0	9·5	10·0	8·0	7·0
Anti-G_2S . .	3·0	7·0	8·5	9·7	9·7	9·5	8·5
Anti-NS . .	4·0	7·5	8·0	8·5	8·5	8·0	8·0
Anti-TBS . .	5·0	7·0	7·0	7·0	7·0	7·0	7·0

The inhibition values for the TBS and anti-TBS system dropped to the level of the MS and anti-MS system. Thus, the inhibitory effects on precipitation produced by diazo treatment show a gradient with a peak at the gastrula and neurula stages. These results are in general agreement with the titrimetric analyses reported above.

DISCUSSION

A number of agents are known which modify the chemical activity of proteins by combining with certain free groups on the protein molecule. These reagents are therefore useful in the analyses of the nature of the protein molecule. They have proved instructive in studies of the relationship between native and denatured protein molecules, as in the work of Mirsky & Anson (1935, 1936 *a* & *b*) on sulphydryl and disulphide groups.

Later, some blocking agents were utilized in studies of the protein structure of hormones: for example, the employment of dinitrofluorobenzene by Sanger *et al.* (1951) in the analysis of the insulin molecule. The method was also found to be useful in the analysis of enzymes, as in the determination of the active groups of pepsin (Herriott, 1937) and urease (Hellerman *et al.*, 1943).

Of particular significance to the study reported here is the alteration of toxins by chemical agents. Thus, formaldehyde was utilized by Ramon (1925) in the production of diphtheria antitoxin; treatment of the diphtheria toxin with dilute formaldehyde resulted in the destruction of toxicity while the antigenic properties of the protein were retained. The possibility of changing the biological activity of the antigen without a loss of its serological properties has been of great importance in prevention of the disease. The detoxication by formaldehyde brought to the attention of numerous immunochemists the value of such

reactions for the study of toxicity and serological activity at the molecular level. To this end, the reactivity of many chemical agents such as ketenes, isocyanate, etc., have been tested on the protein molecule with the idea of elucidating their structure and function (Herriott, 1947).

Evidence suggested that detoxication by formaldehyde is chiefly dependent on free amino groups, e.g. the epsilon amino group of lysine. It became apparent that in an alkaline medium (pH 8·1), not only the amino group but also the indol, phenol, amido, and guanidyl groups were blocked, although the relation of these groups to toxicity is not fully understood. On the other hand, the free phenol groups of tyrosine seem to be significant in the serological activity of the toxin or toxoid.

It was noted by Landsteiner (1945) that inhibition tests could provide a means for determining the specific reactive groups in antigens of unknown composition. It was the hope of realizing this objective that led to the present policy.

In the serological studies of embryonic development in amphibians reported in this series, antisera against embryonic fractions demonstrated significant inhibitory effects on morphogenetic events of both whole embryos and tissues cultured in the media. The Coons (1955) fluorescent antibody method has also indicated a stage specific localization of the antigens, the character of which will be discussed in subsequent reports. A key point in the interpretation of these serological events seemed to be the specific nature and distribution of the active groups on the antigenic molecule.

For this purpose well-known methods of blocking specific radicals with protein group reagents were selected (Table 1). The nature of these reactions have been detailed in reviews by Herriott (1947) and Olcott & Fraenkel-Conrat (1947).

The utilization of these reagents in the molecular analyses of proteins and their serological activity necessitated consideration of the following facts.

First, none of these reagents are known to block only one group. For example, even *p*-chloromercuribenzoate does not react exclusively with SH groups. However, it may be said to be a preferential blocking agent for SH. To a greater or less extent the same may be said of the other reagents used.

The inhibition of the precipitin reaction following blocking of one of these groups, for instance SH, is presumed to be the result of a stereochemical change on the surface of the protein molecule. At the same time the extent of the inhibition may not be linearly related to the number of specific groups blocked.

The position of a specific group on or in the molecule under study will influence the response to the specific reactive agent employed. Such differences must be presumed to result in differences in both titratable and serological response to the blocking agents.

According to data from monolayer-tests of the antigenicity of ovalbumin (Yamaoka, 1952), the number of the diazotizable groups on ovalbumin increases in proportion to the degree of purification of the protein. Thus, it is

evident that the efficacy of the application of reactive agents in analysing specific groups on antigens depends on their purity. It must be recalled that the embryo fractions used in this study are complex antigenic mixtures, so that variation must be expected in the data depending upon the reagents used and interpretation must be very cautious.

Another problem arises from the possibility of denaturation of the antigens, or change in the activity of the group in question, when the more drastic reagents or procedures are used. This particularly applies in the interpretation of data obtained with the unpurified fractions used here. For this reason drastic reagents were avoided wherever possible and, when they are used, the results were taken into consideration only for supplementing other data.

Another important question which must be considered is the configuration of the macro-molecules, and the relation of this configuration to the availability of the reactive groups to the blocking agent. However, little information is to be found on this subject.

In this analysis of the stage specificity of antigenicity and its relation to morphological and cytochemical processes of cell differentiation, recognition of the complexity of the antigenic materials used is essential. Consequently, an attempt was made to maintain identical conditions in the treatment of each stage with a given reagent and to interpret the serological data within the limits thus circumscribed.

With these limitations in mind, more important aspects of the data collected will be discussed.

The sulphydryl groups

The PCMB titration data suggests that the cleavage stages of the embryo are rich in SH groups and that at the blastula stage a sharp animal-vegetal gradient exists in the *S*-fraction but not in the *V*-fraction. This is followed by a loss of the gradient during the period of active cell migration occurring at gastrulation, a process which leads to a marked redistribution of chemical entities. The results of this redistribution become evident at the neurula stage when a dorsal-ventral sulphydryl gradient seems to have become established. There was a slight indication that a ventral-dorsal gradient exists in the *V*-fraction of the gastrula but little evidence of such a gradient is noted in the neurula stage. With further development a decrease in titratable SH groups was observed, but these values remained higher than those found in adult tissues. These titrimetric observations confirm the cytochemical findings of Needham (1950), who suggested that a significant event of blastulation must be related to the animal-vegetal gradient of sulphydryl groups detected by the sodium nitroprusside reaction. In this regard Brachet (1957) has detected the —SH containing proteins accumulated at the animal or dorsal side of the amphibian embyro.

These titrimetric data may be compared with the inhibition of the precipitin

systems following treatment of the S-fractions with the —SH reagents PCMB and MIA. There were some irregularities in the data resulting from differences in the effectiveness of the two reagents, but the serological modifications resulting from the reactions of these reagents with sulphydryl groups can be summarized as follows.

The involvement of sulphydryl groups in the antigen-antibody reaction becomes detectable in the mature oocyte and gradually rises to a maximum in the soluble components of the morula and blastula stages tested against anti-MS, and then declines to a minor value in the neurula and tail-bud supernatants. This is in essential agreement with the titrimetric determination of SH groups and supports the suggestion that the serological data may be considered as a relative measure of the number of SH groups.

The disulphide groups

The serological activity of the disulphide groups has been inferred by analyses following treatment with cysteine. In summary of the data presented in the results, some serological involvement of disulphide groups is found in the mature oocyte stage, then through the morula stage the inhibition values rise to the same grade observed following treatment of SH groups. At this time a dissimilarity between —SH and —S—S— curve becomes evident. The sharp drop in —S—S— activity observed at the blastula stage seems to indicate a transformation of —S—S— into SH between the morula and blastula stages. Gastrulation is accompanied by a second rise, followed by a characteristic slow decline of —S—S— activity.

This series of tests suggests reduction of the SS-components in the protein complex during the period between the morula and the blastula, when cell division characterizes development. Oxidation of SH groups then occurs at the time of invagination as was indicated by an increase in demonstrable activity of —S—S— groups. Following this peak of activity, the participation of both —S—S— and SH groups gradually decreases throughout the period of development examined.

The question of the nature and function of the SH—SS compounds concerned in these observations is of interest. Specifically, are these groups involved in processes of mitosis, in change of molecular linkages associated with fibre formation or in cellular movement? These questions are unanswerable at the present time. However, it is certain that some change at the molecular level occurs in the *S*-fraction between morula and gastrula stages.

The phenol groups

None of the reagents used can specifically and selectively block the phenol groups, except for tyrosinase which can oxidize some of these groups. However, the determination of phenol group activity was accomplished by comparison of the results following iodination, which binds both phenol and SH groups,

with the data obtained from the reagents which selectively bind SH groups. The results following tyrosinase treatment are less certain but were tabulated, to be

TABLE 14

Serological active groups in S-fractions of early embryonic stages against anti-S-fraction sera

Group: *Antigen × antibody*	—SH	—S—S—	*Phenol*	*Amino*	*Imidazol*
LOS × anti-LOS	+	+	–		
LOS × anti-MS	2+	+			
LOS × anti-G_1S					
LOS × anti-G_2S					
LOS × anti-NS					
LOS × anti-TBS					
MS × anti-LOS	2+	+			
MS × anti-MS	3+	3+			+
MS × anti-G_1S	2+	2+			+
MS × anti-G_2S	+	2+			+
MS × anti-NS	2+	2+		+	+
MS × anti-TBS		2+		+	+
BS × anti-LOS	2+				
BS × anti-MS	3+				+
BS × anti-G_1S	2+	+	2+	+	+
BS × anti-G_2S	2+	+	+	+	+
BS × anti-NS	2+	+	+	2+	+
BS × anti-TBS				+	+
G_1S × anti-LOS	+				
G_1S × anti-MS	2+	+			+
G_1S × anti-G_1S	2+	3+	3+	+	2+
G_1S × anti-G_2S	2+	3+	3+	2+	2+
G_1S × anti-NS	+	2+	2+	2+	2+
G_1S × anti-TBS		+	+	2+	+
G_2S × anti-LOS					
G_2S × anti-MS	2+	+			+
G_2S × anti-G_1S	+	3+	3+	+	2+
G_2S × anti-G_2S	+	3+	3+	2+	2+
G_2S × anti-NS	+	+	3+	2+	2+
G_2S × anti-TBS		+	+	2+	+
NS × anti-LOS					
NS × anti-MS		+			+
NS × anti-G_1S		2+	+	+	+
NS × anti-G_2S	+	2+	3+	3+	2+
NS × anti-NS	+	+	2+	3+	2+
NS × anti-TBS		+		3+	+
TBS × anti-LOS					
TBS × anti-MS					+
TBS × anti-G_1S				+	+
TBS × anti-G_2S		+	+	3+	2+
TBS × anti-NS		+	+	3+	2+
TBS × anti-TBS		+		3+	+

3+, 2+, and + indicate relative degree of activity in antigen-antibody reactions.

used in conjunction with the aforementioned series of tests. On the basis of such an examination the data presented above suggest the existence of moderate

activity of the phenol groups in the large oocyte stage, an almost imperceptible activity in the morula stage and a peak of activity at the gastrula stage, followed by a gradual decline at least as far as the tail-bud stage.

The amino groups

The activity of the NH_2 can be deduced by comparison of the results of DNFB treatment, which blocks the NH_2, SH, and phenol groups, with the data obtained on SH and phenol groups discussed above. Phenyl isocyanate, which blocks NH_2 and SH groups, and benzoyl chloride, which blocks primarily NH_2 groups, were also considered in this respect. These data agree in suggesting that the activity of NH_2 is comparatively low in the *S*-fractions prior to the gastrula stage. The modification of serological activity following treatment with these reagents rose following gastrulation and culminated in the neurula and tail-bud stages. Since such an increase in activity was not observed in studies of the SH and phenol groups, it must be concluded that it is in these stages that the amino groups assume a major significance.

The imidazol groups

The curve representing the serological reactivity of the imidazol groups may be derived by a comparison of the effects of treatment with diazonium compounds which react with imidazol, phenol, and some indol groups, with the curve obtained for phenol group activity.

The influence of diazonium compound treatment is low in the large oocyte, and shows a slow increment with successive stages, reaching a peak in the gastrula. However, this peak is identical with that of phenol and cannot be specifically attributed to imidazol groups. It can be inferred that there is no characteristic peak of stereochemical activity of the imidazol groups, but rather a continuing rise concurrent with progressive stages of embryogeny.

These results are summarized in Table 14.

SUMMARY

1. Using the embryos of *T. pyrrhogaster*, the activity gradients of serologically active surface groups of protein molecules were analysed in buffered saline soluble (*S*) fractions of early embryonic stages against anti-*S*-fraction sera. Titrimetric methods were utilized in determination of the relative degree of participation of each group in the antigen-antibody reaction, as evidenced by inhibition of precipitation subsequent to inactivation of specific surface groups.

2. Use was made of the blocking reagents *p*-chloromercuribenzoate, monoiodoacetamide, cysteine, iodine, tyrosinase, 2,4-dinitrofluorobenzene, phenyl isocyanate, benzoyl chloride, and diazonium compounds in evaluating the reactivity of the sulphydryl, disulphide, phenol, imidazol, and amino groups.

3. In the *S*-fractions, low activity of SS—SH groups and of the phenol groups is perceptible in the large oocyte; after fertilization the activity of all groups

increases rapidly. The curve of SH activity culminates in the blastula; that of SS shows perceptible activity in the morula, wanes in the blastula, and then again rises to a maximum in the early gastrula. The gradient of activity of the phenol groups reaches its peak in the late gastrula stage. The amino and imidazol groups demonstrate maximum activity in the neurula and tail-bud stages of development.

RÉSUMÉ

Groupes sérologiques actifs chez les embryons des Amphibiens

1. A l'aide d'immunsérums (anti-*S*), on analyse le degré d'activité des groupes de surface sérologiquement actifs des molécules protéiques dans les fractions saline-solubles (*S*) des stades embryonnaires précoces de *Triturus pyrrhogaster*. La détermination du degré relatif de participation de chaque groupe dans la réaction antigène-anticorps est faite par des méthodes titrimétriques traduisant l'inhibition de la précipitation après inactivation de groupes spécifiques de surface.

2. Pour évaluer la réactivité des groupes sulfhydrile, disulfure, phénol, imidazole et amine, on utilise les inhibiteurs suivants: *p*-chloromercuribenzoate, monoiodoacétamide, cystéine, iode, tyrosinase, 2, 4-dinitrofluorobenzène, isocyanate de phényle, chlorure de benzoyle et des composés de diazonium.

3. Dans les fractions *S* de l'oocyte avancé on note une faible activité des groupes SS — SH et phénols; après la fécondation l'activité de tous les groupes augmente rapidement. La courbe de l'activité des groupes SH est maxima au stade blastula; celle des groupes SS montre une activité perceptible au stade morula, baisse au stade blastula puis croît à nouveau pour passer par un maximum au stade de jeune gastrula; le gradient d'activité des groupes phénol atteint le maximum au stade gastrula âgée. Les groupes amine et imidazole montrent l'activité maxima aux stades neurula et bourgeon caudal.

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Les effets de la succion de l'un des deux premiers blastomères de l'œuf de Grenouille (*Rana temporaria* et *Rana esculenta*)

par J. MILAIRE[1, 2]

Laboratoire d'Anatomie et d'Embryologie de l'Université de Bruxelles

AVEC DEUX PLANCHES

La destruction d'un blastomère au début de la segmentation de l'œuf d'Amphibien est l'une des plus anciennes opérations de l'embryologie expérimentale. Après les premières tentatives de Roux, A. Brachet (1904) répéta cette expérience en détruisant au moyen d'une aiguille chauffée, l'un des deux premiers blastomères de l'œuf de grenouille rousse. Les résultats obtenus ont conduit l'auteur à démontrer le développement normogénétique du blastomère sain et à établir l'existence d'une relation constante entre le plan de symétrie bilatérale de l'œuf fécondé et celui de l'embryon quelle que soit l'orientation du premier plan de segmentation.

Des résultats comparables, mais plus nuancés, ont été obtenus sur le même matériel par Vintemberger (1928), qui détruisait le blastomère par une irradiation localisée aux rayons X. Cette méthode paraît être moins nocive que la précédente puisque, après une irradiation faible, le blastomère irradié peut être le siège d'un processus de régulation partielle, vraisemblablement dû à l'empiètement de la moitié saine sur la moitié affectée.

Il a paru opportun de reprendre ces investigations dans un esprit moderne, en évitant l'emploi de moyens susceptibles de diminuer la vitalité du germe, comme c'est le cas pour la piqûre ou l'irradiation. Le meilleur procédé nous a paru être la succion de la partie centrale du blastomère. Lorsque Vintemberger décrivit cette technique, en 1929, son but était de réaliser l'ablation totale ou partielle du noyau et d'étudier ses conséquences sur l'activité plasmodiérétique de la cellule privée de matériel nucléaire. La suppression totale du noyau est suivie de l'arrêt immédiat de tout processus de clivage cytoplasmique et l'œuf évolue dans ce cas vers la formation d'un embryon partiel. L'auteur estime d'autre part que le maintien d'une activité plasmodiérétique réduite ou normale est toujours lié à la persistance d'une partie ou de la totalité du matériel chromosomial.

[1] Aspirant au Fonds National de la Recherche Scientifique de Belgique.

[2] *Author's address*: Laboratoire d'Anatomie et d'Embryologie Humaines de l'Université de Bruxelles, 97, rue aux Laines, Bruxelles, Belgium.

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En étudiant les répercussions plus tardives de cette opération sur le développement embryonnaire, nous avons vu se manifester divers phénomènes encore inconnus dans le domaine des Amphibiens, dont se dégagent notamment quelques nouveautés à ajouter au répertoire des anomalies.

MATÉRIEL ET TECHNIQUES

L'étude a porté sur l'œuf de *Rana temporaria* et, accessoirement, sur celui de *R. esculenta*. Sur un total de 300 œufs environ, opérés au cours des saisons de 1958 et de 1959, dix-huit seulement se sont développés et ont dépassé le stade de la gastrulation. Le nombre impressionnant d'opérations infructueuses doit être attribué à la fréquence avec laquelle s'est produite, après la succion, une éruption accidentelle de matériel ovulaire par l'orifice de pipettage.

La manœuvre expérimentale consiste à introduire l'extrémité effilée d'une pipette de verre dans l'hémisphère animal de l'un des blastomères à divers stades de la première division de segmentation, et à extraire par succion une fraction du matériel ovulaire profond. La pipette est introduite parallèlement au plan de segmentation de façon à pénétrer le blastomère sans léser son congénère. Au moment de l'opération et pendant les jours qui suivent, les œufs sont placés dans une solution d'Elkosine à 2 gr. pour mille dans de l'eau de ville bouillie et filtrée. Leur maintien à une température de 12° C. ralentit suffisamment leur développement pour que des observations quotidiennes permettent d'apprécier les principales étapes de l'ontogénèse.

La formation accidentelle d'un extra-ovat post-opératoire peut survenir brusquement, au cours des premiers cycles de segmentation, et provoquer la mort immédiate de l'œuf; dans certains cas, au contraire, ce processus évolue lentement et finit par se tarir sans affecter la vitalité de l'embryon; il peut enfin survenir, pour des raisons qui restent obscures, en fin de segmentation ou même au cours de la gastrulation.

Nous avons soumis sporadiquement les produits de la succion à la réaction de Feulgen. Ce test démontra assez souvent la présence de substance chromosomiale dans le matériel aspiré mais ce cas est loin d'être constant et bon nombre de déviations de l'organogénèse ont été la conséquence d'une soustraction exclusivement cytoplasmique.

Les embryons obtenus ont été fixés au Bouin et étudiés sur coupes sériées de 10 μ, colorées soit à l'hémalun-éosine, soit au picro-blauschwarz (Romeis, 1948), soit encore par une méthode trichrome combinant l'hémalun, le rouge ponceau et le bleu d'aniline, récemment mise au point par J. Pasteels.

Les numéros des embryons comprennent, de gauche à droite, les initiales de l'espèce utilisée, le numéro d'ordre du cas suivi du millésime de l'année pendant laquelle l'expérience a été faite. Par exemple, le n° RT 58/59 est celui du 58ème œuf de *R. temporaria* opéré en 1959.

Les termes utilisés pour désigner les divers aspects de l'induction neurogène sont ceux qui furent récemment proposés par Dalcq (1957).

RÉSULTATS

Allure générale des observations in vivo

Bien que divers phénomènes post-opératoires demandent encore à être précisés, les principales conséquences immédiates de la succion sur le clivage se dégagent de nos premiers essais.

Lorsque la succion provoque un arrêt de la segmentation du blastomère opéré, sans affecter la vitalité de l'œuf, trois évolutions sont possibles. La plus courante est la segmentation unilatérale et complète du blastomère respecté, aboutissant à la constitution d'une hémi-blastula ou d'une hémi-gastrula. Il est cependant encore impossible de savoir si cette segmentation partielle évolue toujours de façon normogénétique car la presque totalité de ces œufs meurent avant de gastruler ou au cours de la gastrulation. Nous avons observé 76 cas semblables parmi lesquels un seul hémi-embryon s'est formé. La seconde évolution, moins fréquente, est la segmentation unilatérale mais partielle du blastomère respecté; dans la plupart des 24 cas de ce genre qui ont été observés, le pôle végétatif de ce blastomère ne participait pas au clivage et subissait une cytolyse rapide. Aucun de ces œufs n'est parvenu à gastruler. On peut enfin assister, parallèlement au déroulement d'une hémisegmentation de la moitié saine de l'œuf, à une reprise plus ou moins tardive du clivage du blastomère opéré. Dans certains cas, il s'agit d'un simple retard et le clivage reprend pendant les heures qui suivent l'intervention. Dans d'autres cas, au contraire, le réveil du clivage dans le territoire indivis évolue lentement et progressivement à partir de la zone segmentée: l'allure du processus évoque donc un empiètement graduel des mitoses par colonisation nucléaire du matériel indivis. Ce phénomène, dont on imagine tout l'intérêt, devra cependant être précisé lors de nos prochains essais.

Lorsque la succion n'entrave pas la segmentation du blastomère opéré, seul un retard global de leur développement permet de distinguer les œufs expérimentaux des œufs normaux du même lot. Les anomalies qui surviennent dans ces cas apparaissent à un stade avancé de l'organogénèse et, en général, elles n'ont pu être décelées qu'à l'analyse microscopique. Dans de nombreux cas cependant, une lésion corticale situe pendant un certain temps l'endroit du pipettage; souvent ce repère devient indiscernable au moment de la gastrulation, parfois il persiste au milieu d'une petite plage indivise.

Description des résultats

Parmi les dix-huit embryons obtenus, douze présentent d'importantes malformations morphologiques et six se sont développés tout à fait normalement. Les douze anormaux ont été provisoirement groupés en deux catégories selon le moment d'apparition de la manifestation anormale au cours du développement et selon sa compatibilité avec la vie larvaire.

Anomalies apparaissant précocement et s'opposant au développement complet de l'embryon

Dans les quatre cas groupés sous ce titre, l'opération a entraîné une désorganisation profonde du système germinal qui paraît liée à une perte importante de matériel ovulaire, soit par l'arrêt des activités vitales du blastomère opéré, soit, dans un cas de segmentation bilatérale, par la formation d'un important extra-ovat au cours du développement.

Le développement paragénétique et la constitution monstrueuse de l'embryon RT 122/58 en font un exemple typique de cette catégorie.

Après que la succion eut bloqué la segmentation du blastomère gauche, deux évènements sont venus compliquer le développement primitivement unilatéral de l'œuf. Le premier est l'extension secondaire du clivage au territoire indivis, processus qui avait abouti, au début de la gastrulation, à réduire l'étendue visible de la zone insegmentée à une petite plage située près du pôle végétatif. Le second est l'évolution lente et laborieuse de la gastrulation, au cours de laquelle un abondant matériel profond, indivis et non pigmenté, fut progressivement expulsé par l'élargissement croissant de la lèvre blastoporale. Au seuil de la neurulation, l'œuf était composé de deux parties bien distinctes : un gâteau pigmenté reposant sur une énorme masse blanche indivise représentant le matériel nécrosé et secondairement expulsé. Dès son apparition à la surface du territoire pigmenté, la plaque neurale présentait un contour nettement estompé dans toute la zone troncale gauche. La jeune larve qui se forma ensuite offrait l'aspect extérieur d'un hémi-embryon droit moulé par sa face ventrale et gauche sur une masse vitelline indivise. Le relief peu accusé de l'extrémité céphalique laissait prévoir une régulation imparfaite de cette région.

Les principaux organes identifiables à l'examen microscopique sont schématisés sur la Planche 1. Les structures tronco-caudales sont celles d'un hémi-embryon droit, mais sans cœur ni pronéphros. Surmontant la chorde d'aspect normal, se trouve le tube neural dont la moitié gauche est réduite à une mince lame épithéliale raccordée à l'épiblaste aux abords de l'extrémité caudale (Planche 1, coupe C). Le mésoblaste, présent seulement du côté droit, s'organise en masses pseudo-somitiques tandis que l'endoblaste, volumineux et dépourvu de cavité, est confondu avec un matériel indivis aux limites imprécises. Contrastant avec cette structure troncale simple mais imparfaite, la constitution de l'acromérite apparaît dégradée et complexe. La tête est complètement recouverte d'épiblaste, qui s'est différencié ventralement en une large ventouse (Planche 1, coupes A, B). Trois organes neuraux sont identifiables sur la face latéro-dorsale gauche du massif endoblastique. Deux ébauches cérébrales, l'une massive, l'autre tubulaire, se réunissent vers l'arrière pour former l'axe nerveux troncal; à ce niveau de jonction, la chorde se bifurque en deux branches courtes et inégales (Planche 1, coupe B). Entre les deux ébauches cérébrales principales, on reconnaît un troisième organe neural indépendant, largement ouvert à son extrémité céphalique

(Planche 1, coupe A). Peut-être s'agit-il d'une forte réaction placodique induite par les tubes cérébraux rudimentaires.

En conclusion, la région troncale de cet embryon semble provenir d'un développement normogénétique, quoiqu'imparfait, du blastomère droit mais on s'explique difficilement la constitution si bizarre de l'acromérite. La coexistence d'un dédoublement cérébral et d'une bifurcation chordale évoque une division du matériel préchordal, dont chaque contingent aurait été incapable d'induire un cerveau normal. N'est-ce pas là une conséquence de l'utilisation de matériel tardivement colonisé par envahissement nucléaire?

Nous ne ferons que mentionner dans cette note les trois autres cas de la série; les anomalies qui les caractérisent sont de la même importance que celle qui vient d'être envisagée.

Le premier (RT 101/58) est un hémi-embryon gauche qui s'est formé à la suite d'une succion létale du blastomère droit. Comme pour le cas précédent, le clivage s'est progressivement étendu à la partie indivise, probablement par empiètement graduel des mitoses sur le territoire cytoplasmique. L'examen des coupes révèle qu'il s'est visiblement produit une évolution normogénétique du blastomère gauche, mais avec cette particularité remarquable que la moitié droite de la tête s'est constituée.

Le second cas (RT 110/58) est une hypomorphose généralisée et compliquée d'une ébauche de *spina bifida* troncal, résultant d'une succion du blastomère dorsal. La segmentation des deux blastomères s'est poursuivie normalement jusqu'au stade XVI, puis le clivage s'est ralenti au voisinage de la zone lésée. Ce processus fut responsable du *spina bifida*, en affectant la cinétique gastruléenne, mais il fut associé à une déchéance profonde des matériaux organisateurs, lésion plus directement liée à la soustraction cytoplasmique expérimentale.

Le dernier cas (RT 53/59) fut également le résultat d'une succion du blastomère dorsal, mais sans répercussion, ni sur le clivage, ni sur la gastrulation. Au cours de ce dernier processus, l'expulsion spontanée d'un gros extra-ovat vint compliquer la situation, sans entraver cependant le déroulement de la cinétique gastruléenne. On peut supposer que cet accident provoqua une désorganisation de la voûte archentérique: sans qu'aucun signe extérieur de neurulation se soit manifesté, l'œuf évolua vers la formation d'un embryon complètement dépourvu d'acromérite d'une part, d'organes axiaux tronco-caudaux d'autre part.

Anomalies de manifestation plus tardive, compatibles avec la vie larvaire et résultant d'une succion n'entravant pas la vitalité du blastomère opéré

Six résultats répondent aux critères énoncés dans ce titre: dans chaque cas le blastomère opéré à continué à segmenter et il a participé à la formation de l'embryon; dans chaque cas aussi, une malformation affecte tardivement un organe isolé ou un groupe d'organes, sans entraver le développement de l'embryon jusqu'au stade larvaire.

Bien qu'il soit souvent difficile d'imaginer comment la soustraction de matériel

ovulaire a suscité l'anomalie, on comprend cependant assez souvent comment cette dernière s'est installée ou quel a été le territoire germinal affecté.

Dans l'ensemble, les résultats attirent l'attention sur la sensibilité particulière de l'acromérite ou de son matériel inducteur à ce genre de traumatisme. Deux d'entre eux révèlent par ailleurs une conséquence opératoire assez imprévue et qui semble plus directement liée à la perturbation cytologique créée au sein du blastomère opéré. Il s'agit d'un trouble de la résorption vitelline affectant tardivement les dérivés du mésoblaste et de l'endoblaste et s'opposant à la différenciation des cellules qui en sont atteintes.

Nous envisagerons successivement quelques résultats parmi les plus typiques de cette catégorie; afin de ne pas alourdir le texte, les principaux rétroactes opératoires ont été inclus dans la légende des figures.

Perturbation profonde de l'acrogénèse (*cas RE 237/58*). La difficulté que présente le repérage du croissant cortical chez *R. esculenta* ne nous a pas permis d'identifier le blastomère opéré. La larve obtenue montre une extrémité céphalique anormalement aplatie par rapprochement de ses faces latérales. Elle est dépourvue d'inflexion stomodéale et porte une unique ventouse médio-ventrale. L'examen des coupes révèle d'importantes perturbations de l'acrogénèse chez un embryon normalement structuré dans ses régions deutencéphalique et tronco-caudale. Les dimensions de l'ébauche cérébrale antérieure sont très réduites et l'on ne distingue qu'un rudiment de vésicule optique du côté gauche. Le prosencéphale surplombe une région ventrale comblée de mésenchyme et de formations placodiques hypertrophiques (Planche 2, fig. 9). Le pharynx est massif et dépourvu de poches branchiales; à son contact, l'épiblaste n'a pas formé d'inflexion stomodéale mais il a développé à cet endroit un volumineux organe adhésif. La région branchiale se caractérise encore par sa pauvreté en mésenchyme qui ne s'est pas condensé en arcs précartilagineux.

En conclusion, toutes les structures de l'acromérite semblent avoir souffert de l'intervention. L'anomalie cérébrale et la réduction du mésenchyme céphalique évoquent une lésion du matériel préchordal; l'atteinte de l'endoblaste, l'absence d'induction du stomodaeum et l'hyperinduction de l'organe adhésif indiquent cependant que la lésion n'a pas été limitée au seul territoire préchordal.

Dédoublement cardiaque (*RT 16/59*). Quelques observations faites *in vivo* doivent être mentionnées pour comprendre la genèse de cette anomalie. Une succion du blastomère droit n'eut aucune répercussion immédiate sur le clivage qui se déroula normalement. Au cours de la gastrulation, on assista à l'expulsion tardive d'un extra-ovat dans une région latéro-dorsale droite de l'œuf. Cet incident n'entrava nullement la formation de l'embryon mais il en persista, jusqu'à un stade avancé de la neurulation, une trace corticale discernable en avant de la plaque cérébrale. Cette plage dépourvue de pigment fut ensuite déportée ventralement, en arrière du stomodaeum, avant d'échapper à l'observation. Le jeune têtard fixé est extérieurement normal (Planche 2, fig. 1) mais son analyse microscopique révèle un dédoublement complet du tube cardiaque et de la

cavité péricardique. Simplement accolées vers l'avant (Planche 2, fig. 2), les deux ébauches symétriques sont séparées postérieurement par un prolongement de la paroi médio-ventrale du pharynx qui s'étend jusqu'au contact de l'épiblaste ventral (Planche 2, fig. 3). Cette membrane pharyngienne atypique se prolonge jusqu'au niveau du diverticule hépatique où chacun des tubes cardiaques reçoit la veine vitelline correspondante.

De toute évidence, cette adhérence localisée de l'endoblaste à l'épiblaste résulte de la cicatrisation de la blessure corticale créée par l'expulsion de l'extra-ovat, lésion qui fut observée plusieurs fois au cours de la neurulation. On imagine aisément qu'en recouvrant cette lacune superficielle, le feuillet d'épiblaste s'est accolé au pharynx et s'opposa ainsi à la réunion des deux ébauches mésoblastiques latérales destinées à la formation du tube cardiaque. C'est donc très indirectement que la malformation résulte de la succion du blastomère droit, elle est plutôt une conséquence directe de la formation plus tardive d'un extra-ovat dans une zone de moindre résistance corticale.

Trouble de la résorption du vitellus et hypomorphose unilatérale de l'organe auditif (*RT 58/59*). Bien qu'il montre un important retard de développement vis à vis d'un témoin du même lot, l'aspect extérieur du jeune têtard qui s'est formé après la succion du blastomère droit ne permet pas de soupçonner les profondes perturbations décelées à l'analyse microscopique (Planche 2, fig. 4).

L'anomalie majeure atteint, dans la partie droite de l'embryon, la plupart des dérivés du mésoblaste et de l'endoblaste, depuis la région du deutencéphale jusqu'à l'extrémité caudale. Les structures affectées sont formées de grandes cellules bourrées de plaquettes vitellines et indifférenciées. L'ampleur de la lésion s'accentue progressivement depuis la région auditive, où seul le mésenchyme parachordal est atteint, jusque dans la région troncale où le trouble s'est étendu à toute la partie droite de l'ébauche gastrique, aux myotomes (Planche 2, fig. 6), aux ébauches néphrogènes ainsi qu'aux feuillets cœlomiques.

L'anomalie de la vésicule auditive, la seule qui soit vraiment structurelle, semble bien être une conséquence localisée du trouble général de résorption vitelline. Les dimensions de l'otocyste droit sont réduites de moitié et son épithélium ne présente aucune différenciation régionale, hormis la prolifération des cellules ganglionnaires (Planche 2, fig. 5). Vers le dedans, cette vésicule rudimentaire reste séparée du rhombencéphale par une zone œdémateuse de la leptoméninge, tandis que le mésenchyme qui la sépare ventralement de la chorde renferme, comme nous l'avons vu, de nombreuses cellules chargées d'enclaves vitellines (Planche 2, fig. 7). Ces deux particularités régionales, inexistantes du côté gauche (Planche 2, fig. 8), suffisent à rendre compte de l'hypomorphose de l'organe auditif. On connaît en effet le rôle important qu'il faut accorder au mésenchyme parachordal et au cerveau postérieur dans la genèse de l'oreille interne (Yntema, 1950). L'analyse expérimentale de Yntema a montré que l'induction primaire de la placode est assurée par le mésenchyme parachordal. Lorsque ce processus a suscité l'invagination complète de la vésicule, amenant

notamment sa paroi interne au contact du rhombencéphale, ce dernier exerce alors une influence inductrice secondaire, génératrice du canal endolymphatique. Dans le cas qui nous occupe, il est frappant de voir coexister l'anomalie de l'otocyste et le trouble cytologique du mésenchyme parachordal adjacent. La lésion du foyer primaire est manifestement responsable de l'anomalie auditive mais, par surplus, l'ébauche ainsi affectée n'a pu bénéficier de l'induction secondaire du rhombencéphale, en raison de l'œdème méningé qui la sépare du névraxe.

La raison propre du trouble cytologique du mésoblaste et de l'endoblaste nous échappe entièrement mais le fait qu'il n'a touché que la moitié droite de l'embryon indique clairement qu'il s'agit d'une conséquence directe de l'intervention réalisée sur le blastomère droit.

Trouble de la résorption du vitellus associé à une anomalie structurelle du névraxe (*RT 89/59*). Comme pour le cas précédent, une ponction du blastomère droit n'entrava guère le développement de l'œuf jusqu'au stade larvaire (Planche 2, fig. 10) mais elle provoqua du même côté un léger retard unilatéral de la neurulation.

L'analyse histologique de l'embryon révèle une lésion très étendue des dérivés mésoblastiques du côté droit, rappelant en tous points celle qui caractérise le cas RT 58/59. On observe en effet de grandes cellules anormalement chargées de vitellus au sein du mésenchyme céphalique postérieur, mais en arrière de la vésicule auditive, dans les cellules chordales et dans les myotomes (Planche 2, fig. 12). Aucune de ces structures n'est cependant malformée et, cette fois, le pronéphros est normalement constitué.

Il existe fort probablement une relation directe entre ce trouble généralisé du mésoblaste et l'anomalie purement morphologique qui caractérise la partie droite du névraxe. Au niveau du rhombencéphale et de la moelle tronco-caudale (Planche 2, fig. 12), l'altération consiste en une réduction importante de matériel neural. Dans l'acrencéphale, d'autre part, seule la vésicule optique droite est touchée au point d'être réduite à un rudiment de vésicule primaire (Planche 2, fig. 11).

On peut imaginer que la perturbation cytologique qui affecta de nombreux éléments du chordo-mésoblaste a été la conséquence principale de la succion du blastomère droit. Cette lésion a pu affecter unilatéralement l'intégrité fonctionnelle du matériel neuro-inducteur et l'évocation nettement retardée de la moitié droite de la plaque neurale semble en avoir été la première conséquence. Chez la larve fixée, la coexistence d'une anomalie unilatérale de la moelle tronco-caudale et du trouble de la résorption vitelline dans de nombreuses cellules chordales confirment encore, plus tardivement, l'idée d'une lésion primitive de l'inducteur notogénique.

Cependant, aucune lésion semblable du mésenchyme céphalique ne permet de rendre compte de l'anomalie de la vésicule optique. Il est possible qu'elle résulte d'une lésion directe du neurectoblaste à l'endroit de pénétration de la

pipette. Un minuscule extra-ovat postopératoire était en effet encore repérable dans la zone cérébrale antérieure et droite de la jeune neurula.

Développement normal d'œufs opérés

Six embryons sur les dix-huit survivants se sont développés normalement après une importante soustraction de matériel ovulaire *dans lequel la présence de substance chromosomiale avait été constatée dans trois cas*. La segmentation de ces œufs avait été complète à une seule exception près, celle d'un œuf dont un micromère était resté inerte au stade IV.

Dans deux cas, la soustraction opératoire de matériel ovulaire se compliqua de la formation plus tardive d'un extra-ovat, mais sans répercussion sur le développement.

Aucune anomalie structurelle n'a pu être décelée à l'étude microscopique des six jeunes têtards qui ont été fixés. Seul un léger retard général de développement les distingue de leurs témoins respectifs.

CONCLUSIONS

Les conséquences de la succion de l'un des deux premiers blastomères sur le développement de l'œuf de grenouille apparaissent très différentes des effets généralement obtenus par d'autres manœuvres opératoires visant à supprimer l'activité mitotique de ce blastomère. Ces différences sont étroitement liées au maintien, au sein du blastomère opéré, de conditions vitales favorables à sa participation, à des degrés divers, à l'édification de l'embryon.

Dans les œufs les moins touchés, la succion n'entrave pas le clivage de ce blastomère qui participe donc entièrement à la formation de l'embryon. Dans d'autres cas, la soustraction d'un matériel ovulaire probablement plus riche en nucléoplasme bloque l'activité mitotique du blastomère opéré tout en respectant pendant un certain temps la vitalité des matériaux qui restent en place. Certaines observations qui devront être confirmées nous autorisent à penser qu'un tel blastomère apparemment exclu participe encore partiellement à l'édification du germe grâce à un processus d'extension secondaire du clivage résultant d'une colonisation nucléaire à partir de la zone segmentée.

Lorsque, dans le premier cas, le clivage se poursuit normalement et que des anomalies structurelles apparaissent en des territoires qui dérivent du blastomère opéré, il est indéniable que ces malformations sont la conséquence directe, soit de la soustraction expérimentale de matériel ovulaire, soit de la lésion corticale créée au point de pénétration de la pipette. Dans les six cas répondant à ces critères et groupés dans la seconde catégorie, l'organisation germinale est généralement bien respectée et la perturbation ne se manifeste que tardivement au niveau d'un organe isolé ou des dérivés d'un même feuillet. Les anomalies les plus fréquentes se situent dans l'acromérite qui est affecté à des degrés très variables, depuis un simple vice de position de l'infundibulum (cas RT 98/58, non

décrit) jusqu'à l'hypomorphose complète du cerveau antérieur et du système branchial (cas RE 237/58). Dans deux cas cependant, le trouble majeur n'est pas simplement structurel mais il se manifeste à l'échelle cellulaire par un arrêt ou un important retard de la résorption des plaquettes vitellines au sein du mésoblaste ou de l'endoblaste. Bien que le mécanisme réel de ce trouble reste encore obscur, on saisit mieux ses relations avec la succion expérimentale qui a pu entraîner un déséquilibre dans les rapports existant normalement entre le noyau et le cytoplasme, ou encore priver l'œuf de matériaux indispensables à son métabolisme nutritif. De toute façon, cette anomalie cytologique a altéré les propriétés inductrices de ces cellules, altération dont témoignent, dans un cas une hypomorphose de la vésicule auditive, dans l'autre une lésion de la moitié droite du névraxe.

Une seule exception à ce groupe de résultats doit cependant être mentionnée, il s'agit du cas RT 53/59. Bien qu'il se soit segmenté complètement, cet œuf fut rapidement le siège d'une hypomorphose importante des organes axiaux. Cette déviation précoce du développement est néanmoins survenue après l'émission spontanée d'un abondant matériel ovulaire et elle n'est probablement pas le résultat de la succion initiale.

Les conséquences opératoires se compliquent considérablement lorsque la succion produit un arrêt du clivage du blastomère opéré et que l'œuf évolue donc vers une hémi-segmentation. Les procédés drastiques utilisés par A. Brachet et par Vintemberger (cf. p. 586) pour supprimer l'activité d'un blastomère excluaient toute possibilité d'une participation du matériel détruit à la formation de l'embryon. Il en résulta donc, dans la majorité des cas, un développement normogénétique du blastomère respecté. Le maintien temporaire des activités vitales du blastomère resté indivis peut orienter fort différemment la destinée de l'œuf. Dans le cas RT 98/58, qui ne fut pas mentionné dans cette note, on observa un réveil tardif du clivage au sein du blastomère bloqué par la succion. Il s'agissait donc d'un simple retard de segmentation et l'opération n'entraîna qu'une légère perturbation de l'acrogénèse. Dans la première catégorie de résultats, au contraire (à l'exception du cas RT 53/59), la segmentation s'est progressivement étendue au blastomère indivis à partir du territoire segmenté. Ce processus, que l'on pourrait considérer comme une tentative abortive de régulation, n'aboutit qu'à perturber profondément la cinématique gastruléenne tout en bouleversant complètement le déroulement de la morphogénèse. Au lieu d'évoluer normogénétiquement, les œufs hémisegmentés ont produit des monstres inexplicables dans lesquels toutes les ébauches semblent avoir souffert de l'intervention. Sous réserve de confirmer ce processus au cours d'observations ultérieures on peut déjà supposer que l'extension du clivage par colonisation nucléaire produit un morcellement des territoires présomptifs dans la partie segmentée de l'œuf. Cette désorganisation peut rendre compte de certaines hypomorphoses mais il est cependant étonnant de constater que dans la plupart des embryons monstrueux du premier groupe, la lésion n'a respecté aucun des organes embryonnaires.

SUMMARY

Suction by means of a pipette introduced into one of the two first blastomeres of the frog egg with resultant partial loss of blastomere material, has different effects on the embryonic development of the egg according to whether or not it abolishes the mitotic activity of the manipulated cell.

When the cleavage is unilaterally inhibited, most eggs form half blastulas which generally cannot reach the gastrula stage. Others show a secondary encroachment of micromeres into the undivided area, which is probably due to a nuclear colonization of the inert material. The three resulting embryo monsters obtained show extensive and early malformations expressing a gross germinal disorganization.

When the manipulation does not hamper the complete cleavage of the egg, probably because suction of the blastomere has not resulted in extensive loss of chromosomal substance, more specifically located abnormalities appear at later stages of organogenesis. The acrencephalon is the most frequently affected part of the embryo. However, a cytological disturbance was observed in two cases in which the yolk resorption in the mesodermal and endodermal cells was affected; these injured cells were not differentiated and lost more of their inductive properties.

Six completely normal embryos developed after suction which did not affect the mitotic activity of the operated blastomere.

REMERCIEMENTS

Il nous est agréable d'exprimer notre profonde gratitude à Monsieur le Professeur A. Dalcq, qui fut l'inspirateur de ce travail et ne cessa de s'y intéresser tout en nous faisant bénéficier de sa grande expérience dans le domaine des Amphibiens.

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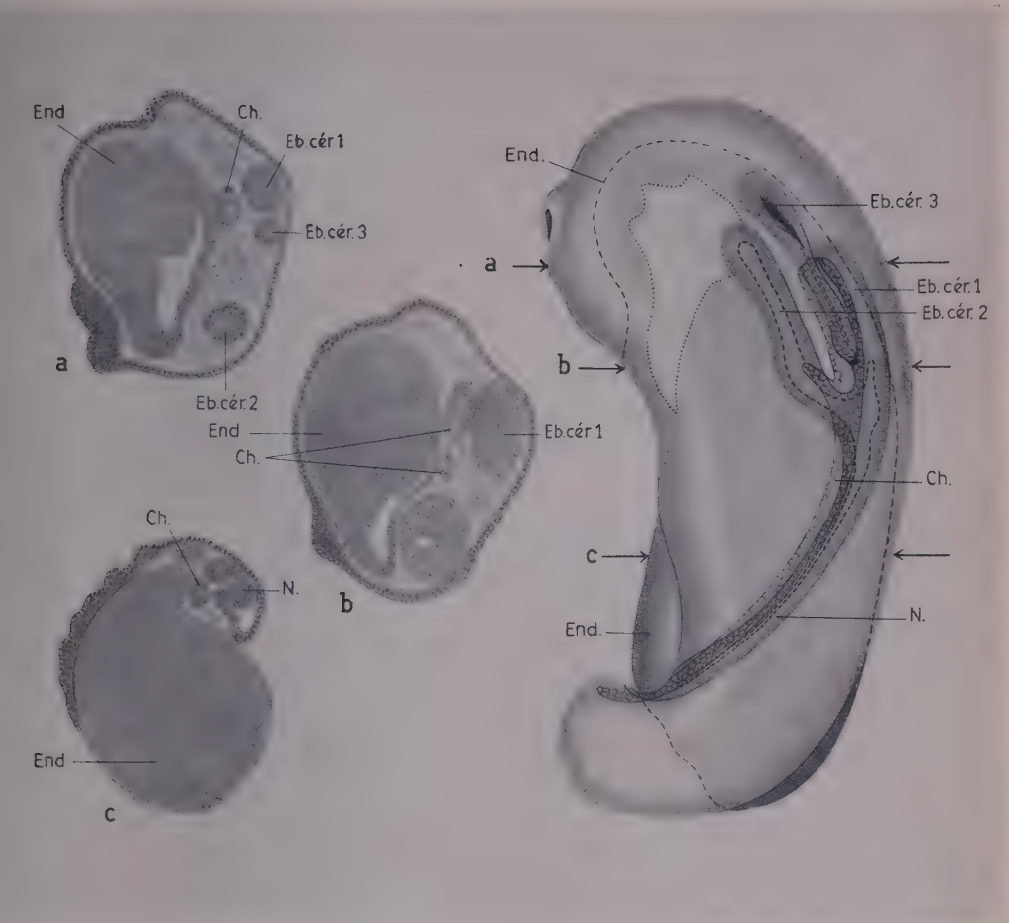
EXPLICATION DES PLANCHES

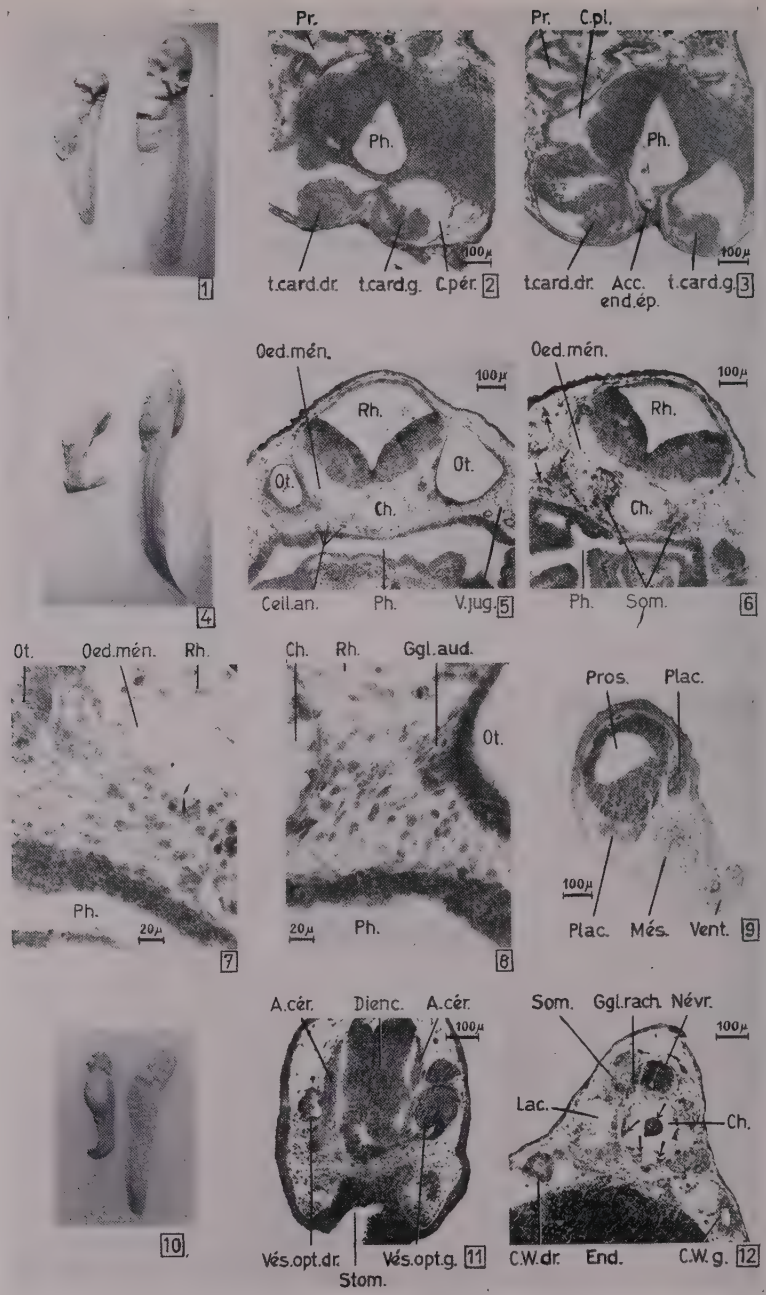
Planche 1

Représentation schématique de l'embryon RT 122/58 en vue latéro-dorsale gauche. Les principaux organes axiaux sont vus par transparence; les limites de la portion invaginée du massif endoblastique sont indiquées par un trait interrompu. La coupe (*a*) intéresse la partie antérieure de l'acromérite et



J. MILAIRE

Planche 1



J. MILAIRE

Planche 2

montre les trois formations cérébrales rudimentaires. La coupe (*b*) intéresse l'étage postérieur de la tête, en avant du niveau de jonction des deux ébauches cérébrales 1 et 2. La coupe (*c*) intéresse l'étage postérieur de la région troncale. *Ch.*, chorde; *Eb. cér. 1*, ébauche cérébrale dorsale, dépourvue de cavité; *Eb. cér. 2*, ébauche cérébrale ventrale creuse; *Eb. cér. 3*, ébauche cérébrale intermédiaire, ouverte en avant et creuse en arrière; *End.*, endoblaste; *N*, névraxe.

Planche 2

Toutes les figures sont des photographies non retouchées prises sur sheet-films Agfa Isopan ISS.

Fig. 1. A gauche, embryon RT 16/59 et à droite, un embryon normal issu du même lot d'œufs fécondés. La taille réduite de l'embryon expérimental et la croissance moins prononcée de ses branchies externes témoignent sur ce cliché de son retard général de développement vis à vis de l'embryon témoin. Rien ne permet cependant de suspecter, à l'examen extérieur, l'anomalie cardiaque dont cette larve est atteinte.

Fig. 2. Coupe transversale, intéressant la partie crâniale de la cavité péricardique de l'embryon RT 16/59 (coloration trichrome). Les deux tubes cardiaques rudimentaires sont accolés par leur face interne et sont reliés au péricarde pariétal par un méso dorsal et un méso ventral.

Fig. 3. Coupe transversale, intéressant la partie caudale de la cavité péricardique de l'embryon RT 16/59 (coloration trichrome). Une double membrane résultant de l'accolement de la paroi médio-ventrale du pharynx avec l'épiblaste se trouve interposée entre les deux ébauches cardiaques rudimentaires. Remarquer également l'aspect anormalement dilaté des tubes pronéphritiques, malformation dont la cause n'a pas pu être déterminée.

Fig. 4. A gauche, embryon RT 58/59 et à droite, une larve normale issue du même lot d'œufs fécondés. L'aspect de l'embryon anormal rend compte du retard important et général de son développement par rapport au témoin. A l'exception d'une incurvation caudale qui n'a pas pu s'expliquer par l'analyse histologique, aucun signe extérieur ne permet de soupçonner les anomalies des organes profonds dont est atteint l'embryon expérimenté. *Rétroactes*: Succion réalisée dans l'hémisphère animal du blastomère droit, à la fin de la première division de segmentation. Il s'en suivit une segmentation normale et complète de l'œuf, puis un léger ralentissement du processus de gastrulation. L'endroit de la piqûre resta longtemps repérable au cours du clivage; il se localisa finalement sur un micromère droit en voie de cytolyse. Les résidus de cette cytolyse se sont progressivement étalés à la surface de l'œuf et chez la neurula, ils recouvraient la partie antérieure et droite de la plaque cérébrale.

Fig. 5. Coupe transversale de l'embryon RT 58/59, au niveau de la région auditive (coloration trichrome). Cette préparation montre l'importante hypomorphose de la vésicule auditive droite (à gauche sur la photo) qui reste séparée du rhombencéphale par une zone œdémateuse de la leptoméninge. Du même côté, le mésenchyme parachordal contient de nombreuses cellules bourrées de vitellus (voir les figures 7 & 8).

Fig. 6. Coupe transversale de l'embryon RT 58/59, dans la région postotique (coloration trichrome). Présence de nombreuses cellules surchargées de vitellus au sein du somite droit, du mésenchyme céphalique adjacent et de la paroi droite du pharynx.

Fig. 7. Détail de la figure 5, au niveau de la région parachordale droite. On aperçoit la paroi de l'otocyste anormal en haut et à gauche, la substance blanche du rhombencéphale en haut et à droite ainsi que la voûte du pharynx en bas. Le mésenchyme parachordal est formé de grandes cellules bourrées de plaquettes vitellines qui entravent, par leur présence, l'observation distincte des limites nucléaires. Remarquer l'œdème méningé au contact du rhombencéphale.

Fig. 8. Détail de la figure 5, au niveau de la région parachordale gauche. L'otocyste est à droite, la chorde à gauche, le rhombencéphale en haut et le pharynx en bas. Les cellules du mésenchyme parachordal droit sont normales, elles ne contiennent que peu d'enclaves vitellines et leurs noyaux sont bien distincts.

Fig. 9. Coupe transversale de l'embryon RE 237/58, intéressant la région céphalique antérieure (coloration au picro-blauschwarz). Sous le prosencéphale d'aspect rudimentaire, on peut voir un territoire mésenchymateux comblé de formations placodiques hypertrophiques. La zone médio-ventrale de l'épiblaste est épaissie en une volumineuse ventouse impaire, à l'endroit où ce feuillet s'infléchit normalement en stomodaeum. *Rétroactes*: La succion a été réalisée à la fin de la première division de segmentation. Il s'en suivit un clivage normal et complet de l'œuf mais la blastula ainsi formée entra en gastrulation avec un retard considérable de plusieurs jours. La malformation qui affecta l'acrogénèse ne fut constatée que lors de l'examen extérieur de la larve, fixée le 10ème jour de son développement.

Fig. 10. A gauche, l'embryon RT 89/59 et à droite, une larve normale issue du même lot d'œufs fécondés. L'embryon expérimental montre un retard global de développement vis à vis du témoin non opéré. *Rétroactes*: Une succion du blastomère droit, réalisée à la fin de la première division de segmentation, n'eut aucune répercussion immédiate ni sur le clivage, ni sur la gastrulation. La lésion

corticale expérimentale resta longtemps repérable et se situait chez la jeune neurula en lieu et place de la plaque cérébrale droite. Le relief neural est en effet apparu, du côté droit, avec un retard de plusieurs heures sur le soulèvement de la moitié gauche de la plaque neurale. Il s'agit là du seul fait anormal qui fut observé *in vivo*.

FIG. 11. Coupe transversale de l'embryon RT 89/59, intéressant la région diencéphalique (coloration trichrome). La vésicule optique droite (à gauche sur la photo) présente un aspect franchement rudimentaire par rapport à la même ébauche du côté gauche. Aucune placode cristallinienne n'a pu être décelée au voisinage de l'ébauche visuelle droite. Noter l'abondance des éléments sanguins présents dans la lumière des vaisseaux cérébraux artériels; cette observation semble traduire une importante stase circulatoire mais cette hypothèse n'a cependant pas pu être vérifiée.

FIG. 12. Coupe transversale de l'embryon RT 89/59, intéressant la région troncale (coloration trichrome). La préparation montre la répartition des cellules surchargées de vitellus au sein des dérivés du chordo-mésoblaste. Le trouble est particulièrement évident au niveau de la chorde et du canal de Wolff droit. Remarquer que le somite droit est creusé de curieuses lacunes qui ne semblent pas affecter la forme générale de l'ébauche. Ces vastes espaces lacunaires se rencontrent dans la plupart des somites droits et sont donc une autre conséquence de la succion du blastomère droit.

Liste des abréviations. Acc. end. ép., accolement endo-épiblastique; *A. cér.*, artère cérébrale; *Cell. an.*, cellules anormales, surchargées de plaquettes vitellines; *Ch.*, chorde; *C. pér.*, cavité péricardique; *C. pl.* cavité pleurale; *C. W. dr., g.*, canal de Wolff droit, gauche; *Dienc.*, diencéphale; *End.*, endoblaste; *Ggl. aud.*, ganglion auditif; *Ggl. rach.*, ganglion rachidien; *Lac.*, espace lacunaire; *Més.*, mésenchyme; *Névr.*, névraxe; *Oed. mén.*, œdème méningé; *Ot.*, otocyste; *Ph.*, pharynx; *Pr.*, pronéphros; *Plac.*, placode; *Pros.*, prosencéphale; *Rh.*, rhombencéphale; *Som.*, somite; *Stom.*, stomodaeum; *t. card. dr., g.*, tube cardiaque droit, gauche; *v. jug.*, veine jugulaire; *Vent.*, ventouse épiblastique; *Vés. opt. dr., g.*, vésicule optique droite, gauche.

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